Experimental studies on the ecology and evolution of drug-resistant malaria parasites

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Declaration

I, Silvie Huijben, verify that the work contained in this thesis is my own and of my own thought and origin. However, scientific research is of a collaborative nature. The role of my coauthors on each of the studies was as follows.

Chapter 2: William Nelson suggested and developed the selection coefficient metholodology and suggested the density-infectivity analysis. Andrew Wargo was involved in initial planning of the experiment, which built on his earlier work. Derek Sim assisted with experimental sampling and logistics. Damien Drew designed and assisted with the quantitative PCR assays.

Chapter 3: Brian Chan assisted with experimental sampling and logistics.

Chapter 4: Derek Sim conducted experimental sampling, quantitative PCR and analysis of blood smears.

Chapter 5: Derek Sim assisted with logistics, experimental sampling and quantitative PCR assays.

Chapter 6: Derek Sim and Danielle Tomassello assisted with experimental sampling and logistics. Nicole Mideo was involved in the experimental design and applied her within-host mathematic model to inform choice of treatment regimes.

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State College, 17 December 2009

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List of abbreviations

ACT	artemisinin-based combination therapy
ANOVA	analysis of variance
DHFR	dihydrofolate reductase
DMSO	dimethyl sulfoxide
i.p.	intraperitoneal
IPTp	intermittent preventative therapy during pregnancy
LD	linkage-disequilibrium
PABA	para-amino benzoic acid
PBS	phosphate buffered saline
PI	post-infection
qPCR	quantitative polymerase chain reaction
RBC	red blood cell
SP	sulphadoxine-pyrimethamine
WHO	World Health Organization

Abstract

Drug resistance is a serious problem in health care in general, and in malaria treatment in particular, rendering many of our previously considered 'wonder drugs' useless. Recently, large sums of money have been allocated for the continuous development of new drugs to replace the failing ones. We seem to be one step behind the evolution of antimalarial resistance; is it possible to get one step ahead? Are interventions which slow down the evolution and spread of drug-resistant malaria parasites achievable? In this thesis, I address these issues with experimental data, using the well-established rodent malaria model *Plasmodium chabaudi* to understand the selective advantages and disadvantages drug-resistant parasites endure within a vertebrate host and the selective pressures various drug treatment regimes exert on these parasites.

Competitive interactions between drug-resistant and drug-sensitive parasites were observed within the host, with resistant parasites having a competitive disadvantage in the absence of drug treatment. The frequency of resistant parasites at the start of the infection was an important determinant of the strength of selection: the lower their frequency, the stronger the competitive suppression in non-treated hosts and the greater their competitive release following drug treatment. Genetically similar genotypes, one resistant and one sensitive, showed similar dynamics following drug treatment. Multiplicity of infection did not have an effect on the within-host dynamics: a larger number of co-infecting susceptible genotypes did not lead to greater competitive suppression or release of resistant parasites. Lastly, various drug treatment regimes were compared. Conventional drug treatment resulted in the greatest selective advantage for drug-resistant parasites, while less aggressive treatments were equally as effective, or even better, at improving host health and reducing overall infectiousness.

These studies demonstrate that altering the within-host ecology of drug-resistant parasites by administering drugs and hence removing the drug-sensitive competitors has a large influence on the transmission potential of drug-resistant parasites. Furthermore, this thesis provides proof of principle that other drug treatment regimes different from those currently in use could better control drug-resistant parasites, without compromising other treatment goals. In the case of malaria, less drugs may mean extending the useful lifespan of that drug.

1. General introduction

1.1 On kitchen salt and the eradication of malaria

It's the late 1950s and the prospect of highly-effective malaria control and possibly even malaria eradication is in sight. The incorporation of antimalarial drugs to common kitchen salt (known as Pinotti's method) is going to make an end to all suffering from this devastating tropical disease (Pinotti 1954; Payne 1988). Now, five decades later, malaria remains one of the leading causes of death worldwide from a single infectious agent (WHO 2008).

The various medicated salt projects carried out globally have taught us that this approach is not suitable to control malaria. Drug-resistant malaria parasites have prospered in many of the areas where medicated salt was distributed, particularly when pyrimethamine was administered; at times, these projects even resulted in an increase in *Plasmodium falciparum* infections (Verdrager 1986; Payne 1988). However, some early successes were achieved: in Guyana, Iran and Surinam (Bruce-Chwatt 1980). In 1955, the much more effective Global Malaria Eradication Program was launched , using mostly DDT but also chloroquine treatments. These eradication programs, based on intense vector control, were largely responsible for the eradication of malaria from Europe, most of North America, Australia, large parts of South America and several countries in the Middle-East and Eastern Asia by the early 1970s (Bruce-Chwatt 1980). Due to economic, political, organizational and insecticide- and drug-resistance problems, malaria eradication programs were converted into malaria control programs or were abandoned completely in the other regions: sub-Saharan Africa, parts of SouthAmerica and Southeast Asia.

Box 1.1 definitions

malaria control: reduction of malaria incidence, ideally until it is no longer a public health problem

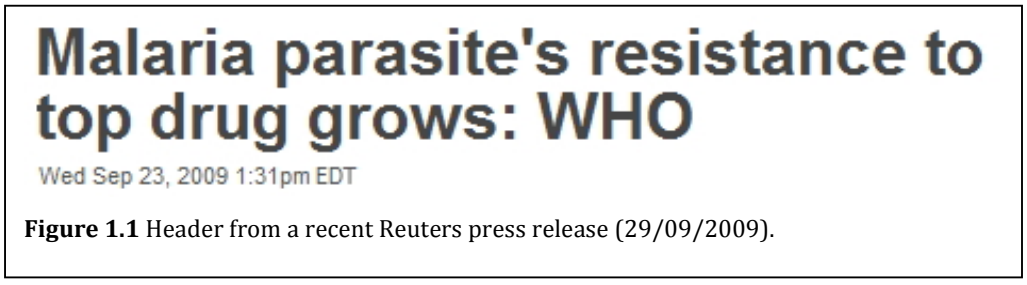
malaria elimination: cessation of regional transmission of malaria

malaria eradication: global extermination malaria transmission

Nowadays, malaria eradication is, once again, firmly back on the agenda (Roll Back Malaria 2008; Feachem and Malaria Elimination Group 2009). The allocation of large

sums of money from the Bill and Melinda Gates Foundation (Grabowsky 2008), the Global Fund to fight HIV, TB and Malaria, the US President's Malaria Initiative and the World Bank's Booster Programme (Mendis et al. 2009) is a great step towards this costly project (Mills et al. 2008). The existing international political commitment is also indispensable in this global undertaking. Nonetheless, malaria eradication is a tremendously ambitious goal (Mendis et al. 2009), which, once started, should not be abandoned. Early desertion of malaria eradication could worsen the situation as a result of drug resistance and reduced immunity in the host population (Trape et al. 1998; Maude et al. 2009). In his instantly-famous speech, Bill Gates concluded *"… we will not stop working, until malaria is eradicated"* (Gates 2007). Whether malaria can be eradicated, particularly elimination from highly endemic sub-Saharan Africa, remains to be seen.

The two most valuable intervention methods proposed in the new eradication programs are the use of insecticide-treated bednets (ITNs) and drug treatment with artemisinin-based combination therapy (ACT) (Roll Back Malaria 2008; Feachem and Malaria Elimination Group 2009). This approach has some striking overlaps with the 1955 eradication program, which was highly dependent on DDT spraying and chloroquine drug use. The former is replaced by pyrethroids for the use on bednets, the latter by ACTs. Unfortunately, resistance against pyrethroids (WHO 2008) and decreased sensitivity to ACTs have already been observed (Figure 1.1; Enserink 2008; Lim et al. 2009).


Malaria parasite's resistance to top drug grows: WHO

Wed Sep 23, 2009 1:31pm EDT

Figure 1.1 Header from a recent Reuters press release (29/09/2009).

For this reason, the malaria eradication proposals incorporate a so-called drug discovery pipeline for the rolling-out of new drugs to replace failing 'old' ones. The costs of the pipeline for research and development alone (i.e. excluding the costs of production and deployment) will be in excess of $US2.5 billion and a further $US1.5

billion will be required every decade that follows (Roll Back Malaria 2008). These are incredible amounts of money for a disease affecting some of the poorest people on the planet. In addition, with failing drugs comes increased morbidity and mortality (Zucker et al. 2003), and a change in treatment recommendation takes time. The question is, are there ways to slow the evolution of drug resistance and thus the drug treadmill (Read and Huijben 2009)?

1.2 Malaria: a global health problem

The figures so often quoted in scientific articles speak for themselves: 3 billion people are currently at risk of a malaria infection, with 250 million cases annually and an estimate of 1 million deaths each year. Malaria is one of the leading causes of childhood death in Africa, with one in five deaths caused by a malaria infection (WHO 2008). Malaria incidence could even be higher than these estimates (Snow et al. 2005). Needless to say, malaria truly is a debilitating health problem, both on an individual and country scale, particularly in the tropical areas where some of the poorest people live. Besides the morbidity and mortality, malaria has a tremendous impact on the economy of malaria-endemic countries. Not surprisingly, poverty and malaria go hand in hand (Gallup and Sachs 2001).

Malaria is caused by protozoan parasites of the genus *Plasmodium*. Four *Plasmodium* species affect human health: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. A fifth species, the zoonotic primate malaria *P. knowlesi*, is able to infect humans as well (Singh et al. 2004). Infections with *P. falciparum* and *P. vivax* cause most of the clinical malaria cases worldwide, with falciparum malaria causing the most mortality, particularly in non-immune individuals (WHO 2008). Malaria is a vector-borne disease and transmitted by female mosquitoes of the genus *Anopheles*. The life cycle of *P. falciparum* parasites is described in box 1.2 and depicted in figure 1.2.

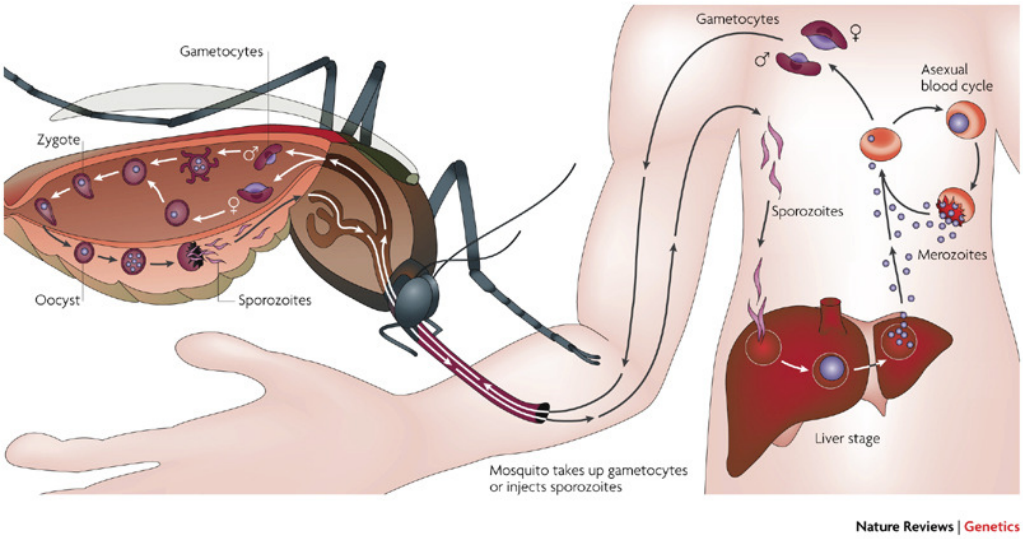


Figure 1.2 *Plasmodium falciparum* life cycle. For details, see Box 1.2. Picture from Su et al. (2007).

Box 1.2 *Plasmodium falciparum* life cycle

The *Plasmodium falciparum* parasite life cycle consists of a human vertebrate host and a mosquito host of the *Anopheles* genus for sexual replication. A person gets infected when a malaria-infected mosquito takes a blood meal, and sporozoites from the salivary glands are injected into the blood stream. These sporozoites migrate to the liver, where they develop into schizonts, which produce merozoites. The merozoites travel to the bloodstream, where they invade red blood cells. The parasites are now called trophozoites; this stage is also called the ring stage, due to the morphological resemblance to a diamond ring. Most of the trophozoites will multiply within the red blood cell, developing into schizonts and producing merozoites which can invade red blood cells again (asexual cycle). A small proportion of the trophozoites will develop into gametocytes, which can be ingested by an uninfected mosquito while taking a blood meal (sexual cycle). Once in the mosquito midgut, the male and female gametocytes develop into male and female gametes. These fuse in the insect's gut to form a zygote, which in its turn develops into an ookinete. The ookinete travels across the midgut wall and forms an oocyst in which sporozoites develop. Finally, the oocyst ruptures and sporozoites move to the mosquito's salivary glands, and the process starts all over again (Figure 1.2; Su et al. 2007).

1.3 Drug resistance and malaria

Drug resistance in malaria is common, as it is in a wide variety of pathogens. The evolution of drug-resistant pathogens has caused much concern over the past decades, starting soon after the introduction of the first antibiotics. Resistance has arisen to every antibiotic in clinical use (Levy 2002) and the pharmaceutical industry is in a race against resistance to develop new antibiotics. The evolution of drug resistance against antibiotics has been referred to as a '*crisis*' and '*worldwide calamity*' in leading articles in the field of antibiotics (Neu 1992; Kunin 1993). Parasitic infectious diseases have similar problems with widely-spread, drug-resistant strains, such as in leishmaniasis (Croft et al. 2006) and African trypanosomes causing sleeping sickness (Matovu et al. 2001). Fungal infections, the most well-known of which is *Candida albicans*, can be complicated by drug resistance (White et al. 1998). Furthermore, viral infections, most notably HIV/AIDS, are tremendously difficult to control as a result of multi-drug-resistant strains (Wainberg and Friedland 1998). Finally, cancerous cells are known to develop resistance under pressure of chemotherapy, resulting in many deaths as a result of unsuccessful treatment (Goldman 2003). Thus, malaria is not unique in its recalcitrance to drug treatment.

1.3.1 History of antimalarial drug resistance

Some antimalarial drugs have been around for a long time (Figure 1.3). Though the Incas likely used cinchona bark for many centuries, the Spaniards claim the discovery of its antimalarial properties in the Americas in the sixteenth century. Several centuries later, in 1820, the active compounds were isolated, with quinine being considered the most effective. Some important discoveries in the late 19th century led to great changes in malaria control. In 1880, Alphonse Lavaran was the first to see malaria parasites under the microscope. Seventeen years later, the mosquito was identified as the malaria vector by Italian Giovanni Grassi and British-Indian Ronald Ross. In the same year, Robert Koch discovered that quinine destroyed malaria parasites in human blood. So far, quinine had only been used by the elite, such as European settlers in colonial areas. The drug was used on a self-medication basis or as prophylaxis. In the late 19th century though, Koch coordinated a small-scale mass blood screening and drug administration among plantation workers in New Guinea. Although very successful, it was deemed too costly and hence abandoned. For the coming decades, drug treatment was, once again, considered for the European settlers

only. Malaria control programs were carried out, but solely based on vector control because of the much lower costs involved. In 1934, a new antimalarial compound was discovered: chloroquine. Unfortunately, it was not until late during the Second World War that it gained more recognition and was deemed safe. Chloroquine was much cheaper than quinine, which opened prospects of mass drug treatments. These were carried out in combination with DDT spraying in the well-known Global Malaria Eradication Program, starting in 1955. In the 1970s, however, when it became clear that malaria eradication had failed in many areas, mass drug administrations were abandoned. Yet, chloroquine was now available for many people living in endemic tropical Africa at affordable prices. The result was self-medication, just as the colonial settlers had once done with cinchona bark and quinine. This time, however, it was done on a much larger scale.

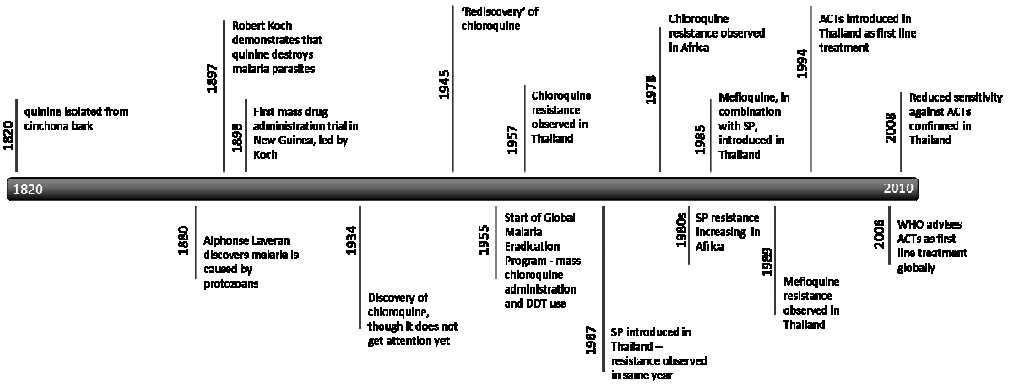


Figure 1.3 Schematic timeline of the major historical events related to antimalarial treatments (Talisuna et al. 2004; Webb 2009). Figure is not drawn on arithmetic scale.

While there are anecdotal reports from 1844 and 1910 of quinine resistance (reviewed in Talisuna et al. 2004), up to the Second World War, when quinine treatment was substituted with other antimalarial drugs, no mass drug resistance against quinine had been observed. Resistance against chloroquine, however, emerged rather rapidly, with the first reports of resistance in Southeast Asia in 1957. Thailand replaced chloroquine as first-line drug in 1973, and other countries in Asia and South America soon followed. In Africa, chloroquine resistance was first found in 1978 and it quickly spread over the continent thereafter. Malawi was the first African country to replace chloroquine as first line treatment in 1993. Resistance to the replacement drug

sulphadoxine-pyrimethamine (SP – Fansidar®) emerged very quickly: less than one year after introduction in Thailand in 1967, resistance was observed. In Africa, SP sensitivity started to decline in the late 1980s. Both chloroquine and SP are now useless in most parts of the world (Talisuna et al. 2004; Hyde 2005). Many other drugs have been introduced over the past six decades, each with the same recurring trend: resistance is usually first observed within a decade after deployment (Figure 1.4; Hyde 2005).

Currently, artemisinin-derivatives are the most effective drugs available. In 2006, the WHO recommended ACTs for first-line treatment globally. Artemisinins work efficiently and fast against parasites, with a very short half-life. Artemisinin-derivatives also affect the transmissible gametocyte stage of the malaria parasite, which most of the other antimalarial drugs do not target. This may have a large impact on malaria transmission intensity (reviewed in Drakeley et al. 2006; Okell et al. 2008a). The antimalarial properties of artemisinin, or qinghaosu as it is called in Chinese medicine, were discovered by Chinese scientists in 1971. The active compounds are extracted from sweet wormwood (*Artemisia annua*). Initially, the parent drug artemisinin was used, but it was soon replaced by the more effective dihydroartemisinin and its derivatives artesunate and artemether (White 2008). The greatest problem with the artemisinin class drugs is that they are much more expensive to manufacture than traditional antimalarials and have a shorter shelf-life. These issues are the most problematic in sub-Saharan Africa (Bloland et al. 2000). Additionally, prices are dependent on supply and demand since the active compounds are extracted from a plant instead of chemical synthesis (Kindermans et al. 2007). As a result of the high and fluctuating prices, the drugs need to be largely subsidised to keep artemisinin-based drugs affordable, ideally at the same price as a course of chloroquine: $0.10-$0.20 (Arrow et al. 2004; Gelband and Seiter 2007).

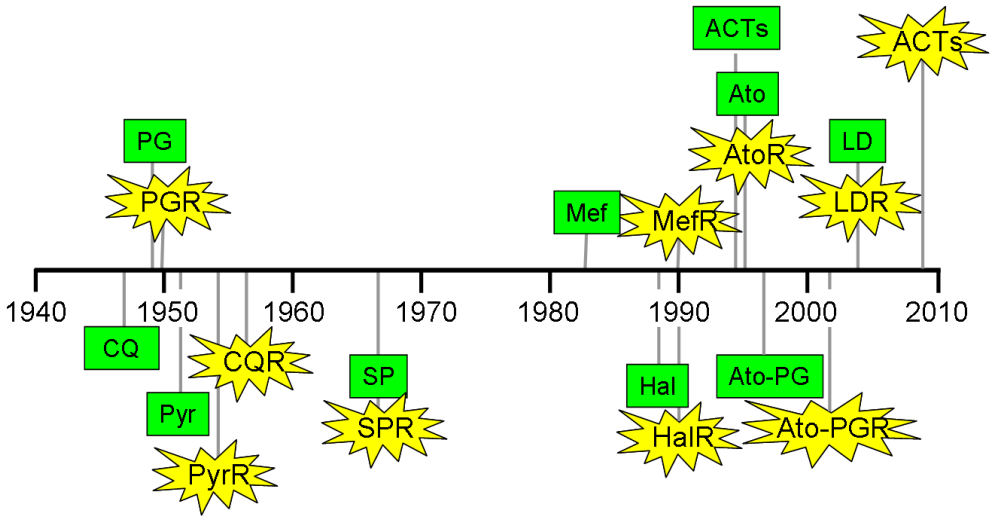


Figure 1.4 History of the introduction of antimalarial drugs (green boxes) and the first detection of resistance in the field (yellow explosion shape). The following abbreviations are used: CQ - chloroquine; PG - proguanil; Pyr - pyrimethamine; SP - sulphadoxine-pyrimethamine (Fansidar®); Mef – mefloquine; Hal – halofantrine; ACTs – artemisinin-based combination therapies; Ato – atovaquone; Ato-PG – atovaquone-proguanil combination (Malarone®); LD – LapDap (chlorproguanil-dapsone). Figure redrawn and adapted from Hyde (2005) and Read and Huijben (2009).

1.3.2 Current problems with drug resistance in malaria

As mentioned above, resistance is widespread against most widely-used antimalarial treatments. Widespread resistance against ACTs would be a very serious global problem, since there are no new front-line drugs waiting on the shelves. Unfortunately, resistance against ACTs is on the rise (Denis et al. 2006; WHO 2007; Enserink 2008; Wongsrichanalai and Meshnick 2008; Carrara et al. 2009; Lim et al. 2009). While treatment efficacy is still very high (over 95%; Carrara et al. 2009), the increased parasite clearance times that are observed are worrisome (Figure 1.5). Reduced parasite clearance and unusually high failure rates are reported for both artemether-lumefantrine (Coartem®) and artesunate-mefloquine (WHO 2007), although reduced clearance in the former is likely a result from reduced lumefantrine absorption when taken without fatty food (Denis et al. 2006). Reduced susceptibility to artesunate-mefloquine seems to be a result of resistance against both components of the drug (Wongsrichanalai and Meshnick 2008; Carrara et al. 2009; Lim et al. 2009). An increased *Pfmdr1* copy number is found to be involved in *in vitro* resistance against

both mefloquine and artesunate, though other molecular mechanisms are likely involved (Carrara et al. 2009; Lim et al. 2009).

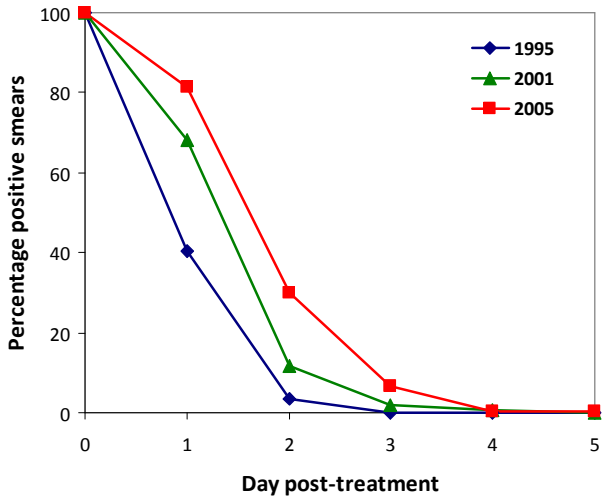


Figure 1.5 Parasite clearance rates (percentage of positive smear counts) on days 1-5 following the onset of a three day treatment of artesunate (4 mg/kg) and a single treatment of mefloquine (25 mg/kg) in patients with *P. falciparum* infections or mixed infections on the northwestern border of Thailand in 1995 (blue line, n=468), 2001 (green line, n=402) and 2005 (red line, n=393). Data from Carrara et al. (2009).

1.4 The evolution of drug resistance

Many factors influence the evolution of drug resistance. The most frequently discussed factors include the cost of resistance, drug pressure and time to *de novo* mutations. However, the spread of drug resistance is not determined by a simple combination of these.

1.4.1 De novo *mutations versus spread of resistant mutants*

The evolution of drug resistance can crudely be divided in two distinct steps. First, drug-resistant parasites arise by a random mutational event of one or a series of mutations in a specific location on a specific gene of the parasite. The chances of getting one such mutation has been estimated in the order of 10^{-9} (Paget-McNicol and Saul 2001). This mutated parasite has to multiply within its host to achieve a high enough density to be transmitted to the next host. I refer to this means of establishment of the mutant parasite as a *de novo mutational event*. The next phase is the transmission phase. The transmitted mutant parasites have to multiply in the new

host(s) and be transmitted again to other hosts. This phase is what I refer to as the *spread of resistance*. Following the initial *de novo* event, much stochastic loss of resistant mutants occurs during the early spreading phase, for instance when the resistant strain is incapable of growing up to large enough numbers in the next host or when the new host is either not bitten by a mosquito or the mosquito does not continue infecting a new person. A resistant strain is considered established once it cannot be lost by chance.

The evolutionary history of resistance against chloroquine and SP tells us that the *de novo* mutational event, or at least the establishment of resistant strains, is a rare occurrence. Globally, resistance against both these drugs has arisen only a handful of times. The subsequent spread of resistance from this handful of events has led to a worldwide drug failure. Resistance against both chloroquine and SP in Africa has never arisen in Africa itself, but originated in Southeast Asia and subsequently spread to Africa (Wootton et al. 2002; Hastings 2004). We know this because of the following.

A single resistant mutant that has spread widely would have resulted in a selective sweep: selection for the resistance gene will result in selection of neighbouring alleles as well. This process leads to a reduction of the genetic variation surrounding the resistance gene. A selective sweep can be measured by detection of linkage disequilibrium (LD): an overrepresentation of certain alleles in combination with the resistance gene is observed, while random assortment of genes in the population would be expected under neutral evolution. The presence of strong LD indicates that there may have been a recent selective sweep. Different selective sweeps result in different variation around the resistance genes and, hence, different origins can be detected. Linkage disequilibrium can decay after long periods of transmission and recombination, but since resistance has arisen relatively recently (within the past 60 years), LD can still be detected in resistant parasites originating from patients from different geographical areas. Therefore, LD provides a great tool to determine the independence of *de novo* resistance mutational events (Wootton et al. 2002; Hayton and Su 2008).

Linkage disequilibrium was found in chloroquine-resistant parasites from Africa and Asia. These resistant parasites shared many more alleles on the chromosome of the

resistance gene than chloroquine-sensitive parasites. Subsequently, at least four independent founder events were demonstrated (Wootton et al. 2002). Using the same method, sulphadoxine-pyrimethamine resistance has been shown to have only a handful of origins, none of which occurred in Africa (Cortese et al. 2002; Nair et al. 2003; Roper et al. 2003; Hastings 2004). However, a more recent study argues for two independent origins in Africa as well (Mita et al. 2009). The different geographic origins and assumed spread of drug resistance against chloroquine and SP is summarized in figure 1.6.

It appears that, at least for these antimalarials, the spread of drug resistance, rather than a multitude of *de novo* mutational events, is the main cause of the widespread resistance currently observed in malaria. This finding implies that our efforts to control drug resistance should focus as much on slowing down the spread of drug resistance as on reducing the likelihood of *de novo* mutations to occur. This is especially true once resistant parasites have been observed in the field. Parasites resistant against chloroquine and against SP have swept remarkably across the African continent; until we understand the factors governing the speed of this process, we will not be able to apply intervention methods to slow it down.

There are exceptions, however, and resistance to some drugs such as atovaquone-proguanil (Malarone®) has occurred from multiple independent origins (Musset et al. 2007). In contrast to chloroquine and SP resistance, high-level atovaquone resistance is conferred by a single nucleotide polymorphism (SNP) that has a major effect on susceptibility to atovaquone-proguanil. Also, resistance against the sulphadoxine component of SP treatment (mutations in *dhps* gene) has arisen multiple times in Africa (Pearce et al. 2009). In contrast, mutations in *dhfr* gene, which confer a much greater resistance against SP than mutations in *dhps* gene, have arisen only a few times as described above. These multiple origins of resistance in *dhps* are likely a result of a weaker selective pressure against additional resistance mutations in an already resistant mutant. Pearce et al. propose that these *dhps*-resistant genes have emerged much more recently than *dhfr*-resistant parasites and chloroquine-resistant parasites. After an equal amount of time under selection, perhaps only one resistant *dhps* parasite strain would predominate as well. However, while resistance to some drugs may occur more regularly as *de novo* mutations than resistance to other drugs, unless

the source of resistant infections is predominantly caused by *de novo* mutations, control on the spread of resistance is still an important measure to control drug-resistant infections.

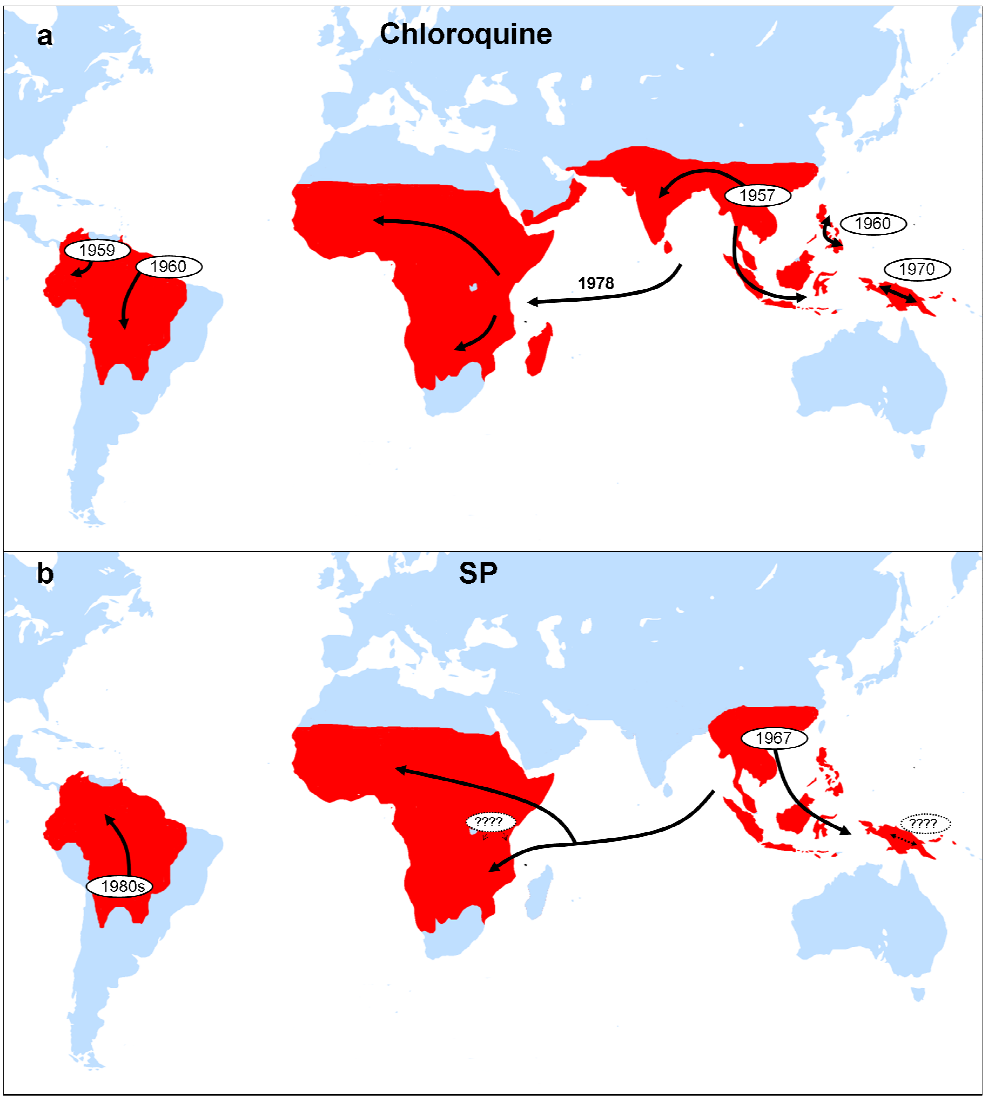


Figure 1.6 The history of chloroquine and high-level pyrimethamine-sulphadoxine (SP) resistance inferred from molecular evolution studies. Chloroquine resistance has spread globally from selective sweeps from five independent origins. Notably, no sweeps began in Africa, where the health burden of drug resistance is greatest. Resistance to SP is tracked by analyses of the *dhfr* gene, which primarily confers resistance to the pyrimethamine component of the drug. The timing of two of the independent origins is unclear. SP resistance may have several local origins in Africa (Mita et al. 2009), but the majority of SP-resistant infections are a consequence of a selective sweep from a single origin in Southeast Asia. The top figure is redrawn from Wellems (2004) and Read and Huijben (2009), the bottom figure is a summary of a large body of studies (Cortese et al. 2002; Nair et al. 2003; Roper et al. 2003; Roper et al. 2004; Maiga et al. 2007; McCollum et al. 2007; Mita et al. 2007; Hayton and Su 2008; McCollum et al. 2008; Saito-Nakano et al. 2008; Mita et al. 2009).

1.4.2 What determines the rate of spread of resistant parasites?

Many factors influencing the rate of spread of drug-resistant parasites have been identified, such as drug use practices, drug half-life, transmission intensity, clone multiplicity, parasite density, host immunity, within-host dynamics and the genetic basis of drug resistance. Many of these are incorporated in theoretical models (Curtis and Otoo 1986; Cross and Singer 1991; Hastings 1997; Mackinnon and Hastings 1998; White 1999; Hastings and D'Alessandro 2000; Koella and Antia 2003; Talisuna et al. 2004; Mackinnon 2005; Hastings 2006; Pongtavornpinyo et al. 2008), but inconsistently.

For instance, there is an ongoing debate about whether drug resistance is more prone to develop in areas with high or low transmission intensity (Bloland et al. 2000). Several arguments have been put forward that high transmission intensity results in a greater spread of drug resistance. High transmission areas have a higher clone-multiplicity (Babiker and Walliker 1997; Arnot 1998), which gives an advantage for drug-resistant parasites co-infecting a host with drug-sensitive parasites when the infection is treated (Hastings and D'Alessandro 2000; de Roode et al. 2004; Mackinnon 2005; Wargo et al. 2007; this thesis). Also, high transmission intensity results, by definition, in a higher number of parasites and thus resistant gene copies to be transmitted through the population, which results in more parasites being exposed to drugs compared to low transmission areas at the same drug pressure (Mackinnon and Hastings 1998). However, there are several arguments to suggest that areas of low transmission intensity are more prone to developing drug resistance. For instance, clone multiplicity results in genetic recombination, which means that the breakdown of multi-locus resistance genes occurs less often at low transmission intensity (Talisuna et al. 2004; Mackinnon 2005). Also, competitive suppression of drug-resistant parasites may be more intense in multi-clone environments in untreated hosts (Chapter 5). Moreover, areas with low transmission intensity typically harbour more non-immune individuals, who have a higher parasite biomass (White and Pongtavornpinyo 2003), are more likely to have symptomatic- and, therefore, drug-treated- infections (Talisuna et al. 2004; Mackinnon 2005; Pongtavornpinyo et al. 2008), and have reduced capability to clear drug-resistant parasites (Cravo et al.

2001). Undoubtedly, all these factors, and probably more, affect the spread of drug-resistant parasites, but the net result has yet to be established.

Another unresolved factor in the rate of spread of drug resistance is the influence of drug pressure. Even though it is generally accepted that drug pressure increases the spread of resistance, the type of drug pressure and to what extent it promotes the spread of resistance is subject to debate (Read and Huijben 2009). It is widely agreed that unnecessary drug usage should be limited in order to avoid the emergence and spread of drug resistance. For this reason, drug usage is limited at a population level, often through increased implementation of better diagnostic methods, such as rapid diagnostic tests, to prevent presumptive treatment (Drakeley and Reyburn 2009) and reduced implementation of mass drug treatments. On an individual patient level, however, drug treatment is given to a maximum intensity, with the aim to avoid the evolution of drug resistance. This is an inconsistency in treatment guidelines that, at least on the face of it, makes little evolutionary sense. Maximum treatment imposes maximum selective advantage to any drug-resistant parasites that are present, greatly enhancing their fitness. The logic behind this is as follows.

When mixed infections of highly-resistant and fully-sensitive parasites are drug-treated, the relative fitness of resistant parasites increases simply because of the survival advantage resistant parasites have over drug-sensitive ones. Additionally, diminished competitive suppression by the susceptible parasites following drug treatment can increase the relative frequency of the resistant parasites even further (Hastings 2003). This competitive release allows the resistant parasites to fill up the ecological space left by susceptible parasites. Mixed infections of various genotypes are very common in human malaria infections (e.g. Arnot 1998; Babiker et al. 1999; Smith et al. 1999; Bruce et al. 2000; Jafari et al. 2004; A-Elbasit et al. 2007; Nwakanma et al. 2008; Vafa et al. 2008; Baruah et al. 2009; Soulama et al. 2009), therefore, mixed infections with resistant and sensitive parasites are common as well. Direct experimental evidence on competition between co-infecting *P. falciparum* genotypes, particularly with drug-resistant parasites, cannot be ethically obtained from human infections. However, evidence from field data strongly argues for the presence of between-genotype competition by suppressed population densities of a genotype when other genotypes are present within a malaria-infected host (Daubersies et al.

1996; Mercereau-Puijalon 1996; Smith et al. 1999; Bruce et al. 2000; Hastings 2003; Talisuna et al. 2006; Bousema et al. 2008; Harrington et al. 2009). Additionally, there is considerable direct experimental evidence for crowding in rodent malaria models (e.g. Jarra and Brown 1985; Taylor et al. 1997; de Roode et al. 2004; de Roode et al. 2005a; de Roode et al. 2005b; Bell et al. 2006; Wargo et al. 2007; this thesis), as well as for competitive suppression and competitive release in mixed infections of drug-resistant and drug-sensitive *P. chabaudi* parasites. Drug-resistant parasites were competitively suppressed in the absence of drug treatment; drug treatment led to the competitive release of resistant parasites (de Roode et al. 2004; Wargo et al. 2007). Additionally, competitive facilitation was observed, whereby the resistant parasites increased to even higher parasite densities following release than they would have in the absence of competition (Wargo et al. 2007).

Suggestive evidence for competitive release and facilitation has been observed in the field, where the effect of SP intermittent preventative treatment for pregnancy (IPTp) on resistance alleles was studied (Harrington et al. 2009). Since some women opt out of using IPTp, they can be used as a 'control' group, providing a unique opportunity to study the effect of drug treatment on human malaria infections. The study found that IPTp use was associated with increased frequency of resistance alleles in the infection. More strikingly, women using IPTp harbored a higher parasite density than women that did not use IPTp (Figure 1.7). Additionally, increased transmission success for multidrug-resistant parasites was observed following combination therapy with chloroquine and SP (Hallett et al. 2006). These results suggest competitive release and perhaps facilitation occurs among drug-treated *P. falciparum* infections when resistant parasites are present.

Thus, drug treatment provides a great advantage to drug-resistant parasites, not only by means of a survival advantage, but also through competitive release. This latter effect may be the more powerful selective effect, playing an important role in the spread of drug resistance when mixed infections dominate, as they do in most malarious areas.

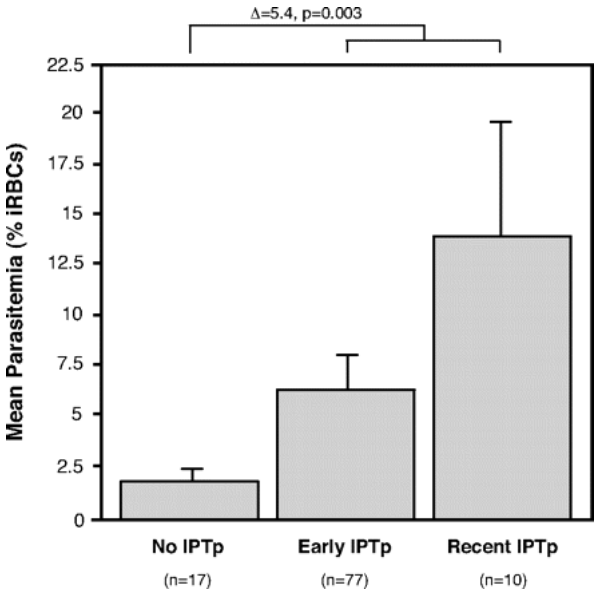


Figure 1.7 IPTp selects for increased level of parasitaemia. Placental parasitaemia was significantly higher in women who received any IPTp versus no IPTp (adjusted difference. 4.9%, *P* = 0.03). Figure from Harrington et al. (2009).

Since competitive release is the result of a complete alleviation of competitive suppression, **the question arises: can resistant parasites be suppressed, while still providing a cure for the patient**? Put differently, can the within-host ecology of drug-resistant and drug-sensitive parasite co-infection be utilized to reduce the relative fitness of resistant parasites? Wargo et al. (2007) and Read and Huijben (2009) proposed the hypothesis that lower drug dosages could reach this goal, simply by maintaining competitive suppression on resistant parasites by not eliminating all competing susceptible parasites. Chapters 2 and 6 of this thesis present experimental tests of this hypothesis.

1.4.3 Cost of resistance

Much circumstantial evidence suggests that resistant malaria parasites have a lower fitness than sensitive parasites in the absence of chemotherapy (Walliker et al. 2005; Felger and Beck 2008; Babiker 2009). Suggestive evidence for a cost of resistance comes from progressive increases in drug sensitivity in populations where drug use has been discontinued. This has been seen in Malawi (Kublin et al. 2003; Mita et al. 2003; Laufer et al. 2006), Tanzania (Temu et al. 2006), South Africa (Raman et al. 2008), Thailand (Thaithong et al. 1988) and China (Liu et al. 1995), although there are also areas where a decrease of resistance has not been observed (McCollum et al.

2007; Yang et al. 2007). Seasonal variation in the frequency of resistant alleles in eastern Sudan and The Gambia is also consistent with costs of resistance. When there is low to no transmission during the dry season, and hence few new malaria cases and essentially no drug use, resistance alleles drop in frequency among the chronically-infected patients who subsequently source the next outbreak. During the wet season, when high transmission ensures many new disease cases and hence high drug usage, resistance alleles rise in frequency (Abdel-Muhsin et al. 2004; Ord et al. 2007; Babiker 2009).

As in other pathogens, costs of resistance in malaria presumably arise from the metabolic costs of efflux or detoxification, or reduced biochemical efficiency associated with target site mutations (Hastings and Donnelly 2005); in other words, genetic trade-offs. Most models of malaria drug resistance evolution recognize these costs of resistance, but, if included at all, they are typically taken as a fixed and relatively modest parameter (e.g. a selective disadvantage *s*, so that the fitness of resistant mutant is 1- *s*, where *s* in the order of 0.1 or less). Additionally, it seems as if there is a widely held assumption that these costs can be mitigated by compensatory mutations, as they can be in bacteria and HIV (Levin et al. 2000), so that *s* can drop further through time. Such selection processes might explain some of the sequential mutational steps associated with chloroquine and SP resistance (Hastings and Donnelly 2005; Hastings and Watkins 2005).

However, the cost of resistance is very likely dependent on the ecological environment of the parasite, so can not be represented as a fixed parameter such as '*s*', but rather as a variable. This is because the costs of resistance are a function of the interactions between co-infecting strains within the host. A small metabolic cost influencing the growth of resistant parasites by themselves can be greatly magnified by competition with other genotypes (Read and Huijben 2009). Thus, the cost of resistance could be substantially increased with the force of infection, which is important to include in models on the spread of drug resistance in areas with a high level of multi-genotype infections.

1.4.4 What is so special about Southeast Asia?

The Thai-Cambodia border seems to be the hotspot for the emergence of antimalarial resistance. Not only have chloroquine resistance, SP resistance and mefloquine resistance arisen in this region, but also the first reports of reduced ACT sensitivity originate from Southeast Asia (Wongsrichanalai and Meshnick 2008). What is so special about this region? Once we understand the specific factors that contribute to making the Thai-Cambodian border such a fertile ground for resistant parasites to emerge, interventions can be undertaken to reduce the chances of it happening again with future antimalarials. Unfortunately, the issue is not resolved yet. Several explanations have been suggested. First, there is a high level of drug use in the private sector, with a large abundance of counterfeit drugs (Hall et al. 2006; Newton et al. 2008). Poor ability to diagnose malaria in this region has also been suggested (Wongsrichanalai and Meshnick 2008). Another option that has been put forward is a unique ability of the parasites in this region to develop *de novo* resistance mutations (Rathod et al. 1997).

High level of drug use in the private sector may certainly contribute to the evolution of resistance since easy accessibility to drugs increases drug pressure. However, the use of counterfeit drugs per se does not necessarily result in a selection for resistance. Often, these drugs contain few antimalarial properties (Newton et al. 2008), and thus impose little selective pressure. However, a large abundance of counterfeit drugs does lead to widely-available cheap drugs, and hence high usage, which can still result in overall increased drug pressure if the counterfeit drugs contain some antimalarial properties. Poor ability to diagnose malaria does not necessarily result in increased selection for resistance either. When a patient is falsely diagnosed with malaria and is given drug treatment, selection for resistance is, evidently, only imposed when malaria parasites get exposed to these drugs. Since Southeast Asia has very low transmission intensity, the chance of a malaria infection occurring when there are significant levels of drug in the blood is much smaller than in high transmission intensity areas such as sub-Saharan Africa. Furthermore, in the case of artemisinin drugs, the chance of this occurring is very small since this drugs half-life is in the order of hours, opposed to weeks or even months in the case of the combination drug mefloquine (White 2002). Finally, there is no evidence of a regional existence of 'high mutator strains' of malaria parasites in Southeast Asia.

A more plausible explanation to the high frequency of new resistant mutants arising from this region is the combination of low immunity and high drug usage (Pongtavornpinyo et al. 2008). The intensity of malaria transmission in Southeast Asia is much lower than in Africa, which results in a much lower immune status in individuals in Southeast Asia compared to Africa. Therefore, infections in this region are far more likely to be drug-treated than they are in Africa. Since most infections in this region are symptomatic, most parasites will find themselves in a new host that is (going to be) drug-treated, whereas a resistant parasite in Africa may need to pass through several asymptomatic hosts (by which it can be lost) before it, by chance, is transmitted to a symptomatic host (i.e. small child or pregnant woman). A recent mathematical model showed that via this process, resistant parasites spread more easily in low transmission areas than high transmission areas (Pongtavornpinyo et al. 2008). Furthermore, low transmission areas have a characteristically lower rate of multi-genotype infections. This could additionally contribute to a better performance of resistant parasites caused by a reduced level of competition in untreated infections. However, fewer multi-genotype infections also mean a reduced benefit of treatment.

These explanations are all theoretical speculations and we just do not know why resistant parasites appear so frequently in Southeast Asia.

1.5 Antimalarial treatment regimes

1.5.1 Treatment objectives

The first and foremost objective of treating uncomplicated malaria (symptomatic malaria without signs of severity or evidence of vital organ dysfunction) is defined by the WHO as *"eradication from the body of the infection that caused the illness"* (WHO 2006, pg 7). In other words, drug treatment should be effective until the last malaria parasite is eradicated from the patient. This approach is aimed at reaching the following three objectives for antimalarial treatment (WHO 2006, pg 7).

(i) Improvement of patient health, most importantly by preventing progression to severe malaria and preventing additional morbidity as a result of treatment failure.

(ii) Reducing transmission intensity as a public health goal.

(iii) Control of the emergence and spread of resistance to antimalarial drugs.

In case of severe malaria, however, the foremost goal is to prevent death and the above objectives are secondary.

As discussed in paragraph 1.4.2, drug pressure is the main determinant for the spread of drug-resistant parasites. Therefore, the approach of radical parasitological treatment seems to obstruct objective (iii): control of the evolution of drug resistance (Read and Huijben 2009). The rational for radical parasitological treatment to control the emergence and spread of resistance to antimalarial drugs is as follows. Conventional wisdom is that lower drug dosages select for *de novo* resistance faster because it exposes more parasites to low drug concentrations and leads to a selection for 'tolerant' parasites. These tolerant parasites are fewer mutational steps apart from high-level resistance than fully susceptible parasites (Hastings and Watkins 2006). Also, radical drug treatment minimizes the likelihood of parasites with the relevant mutations to occur by means of reducing absolute parasite numbers (WHO 2006). While both these rationales may be true (although the first is irrelevant in case of high level resistance following single point mutations) they are only relevant to reducing the likelihood that resistant parasites will arise in the first place. Once resistance is present in a population, these treatment objectives likely provide the greatest positive selection for resistance. However, experimental proof of this contention is missing. There is a great need for empirical data on the basic ecological dynamics of drug-resistant parasites in drug-treated infections to inform current treatment guidelines.

1.5.2 Treatment guidelines

Combination therapy is now recommended by WHO for the treatment of falciparum malaria. Combination therapy is defined as combining two drugs with different drug targets. Therefore, synergistic drugs such as sulphadoxine and pyrimethamine (both targeting the folate pathway) are not considered combination therapy. Combination therapy has been shown to reduce the transmission success of resistant parasites in the absence of multi-drug resistance (Hallett et al. 2004; Okell et al. 2008b). The best combination therapy currently available is artemisinin-based combination therapy (ACT), with either artesunate, artemether, artemotil and dihydro-artemisinin as the first compound and another, slowly-eliminated drug as the partner drug. A slowly-

eliminating partner drug is advised so that a shorter treatment course (3 days) can be given, since a 7-day course is more expensive and adherence is expected to be low (WHO 2006). In effect, however, this results in a mono-therapy of the combination drug for at least several weeks once the artemisinin derivatives are eliminated from the body within a couple of days after treatment. Artemisinin derivatives produce a rapid clearing of the parasites in the first days; subsequent complete clearance of parasites is dependent on the partner drug. The following four ACTs are currently recommended: artemether-lumefantrine (Coartem®), artesunate+amodiaquine, artesunate+mefloquine and artesunate+SP. The choice of ACT should be based on the level of resistance to the partner drug in the area. Another, non-ACT, combination therapy that is very effective is atovaquone-proguanil (Malarone®). However, due to its high costs, it is not recommended for deployment in endemic areas and is therefore mostly prescribed for western travellers, both as treatment and prophylaxis.

Partial treatment is strongly advised against by WHO: *"A full course of effective treatment should always be given once a decision to give antimalarial treatment has been reached"* (WHO 2006, pg 27), to avoid the exposure of parasites to less than radical treatment. If treatment fails within 14 days, the infection should be treated with a second-line antimalarial, preferably another ACT. If treatment fails after 14 days, the first-line treatment should be used. First-line malaria treatment should be changed if the treatment failure exceeds 10%. The therapeutic efficacy should be assessed by a treatment follow-up of at least 28 days (WHO 2006).

Despite changes in treatment recommendation from the WHO, treatment practices still appear to be far from what is being advised. Chloroquine continues to be the most widely used drug, with an estimated several hundred million courses in 2006. Also artemisinin monotherapy is widely available nowadays (Yeung et al. 2008), albeit still expensive. Approximately 85 million courses of ACT have been ordered from manufacturers in 2006 (Gelband and Seiter 2007). Thus, while WHO advises otherwise, ACTs are not the mostly used drugs. With increasing funding to subsidize ACTs and reduce their prices, this is likely to change soon.

1.5.3 Resistance management

Current resistance management is aimed at reducing the chance of *de novo* resistance mutational events from happening. General belief is that once resistance has arisen, there is no way of stopping it or slowing it down:

"As effective and robust as the artemisinin drugs are today, it is only a matter of time before genetically resistant strains emerge and spread. However, practical steps can be taken to push that day further into the future. The logic is as follows. In the case of any antimalarial drug, the new development of drug resistance is a rare event: a chance genetic change in a single parasite in a single patient. But once that single malaria parasite generates multiple descendants, the math changes. Now, mosquitoes can acquire resistant parasites from a single individual and transmit them to other people. The subsequent spread of a robust, resistant clone would be similar to the spread of any malaria strain" (Arrow et al. 2004, pg 4).

Also, a mathematical model has shown that the only way of getting rid of drug-resistant malaria is by eradicating all malaria parasites (Maude et al. 2009). This model, whereby resistance is assumed to be already present in the population, predicts a rapid increase in resistance with interventions based on ACT drug treatment. Thus, the only way to control resistance, is by eliminating malaria parasites all together. In other words, resistance is thought to inherently spread once it has arisen. Therefore, current resistance management is focused on pushing the day of the first emergence of resistant strains further into the future.

Combination therapy is a great tool to reduce the chance of resistant parasites from arising and therefore extending useful lifespan of a drug. The logic is as follows. The mutation rate of *P. falciparum* is estimated to be 10^{-9} (Paget-McNicol and Saul 2001). However, a malaria patient may typically harbour 10^{10}-10^{12} parasites. Therefore, by chance alone, 10-1000 parasites may be resistant against the drug at the time of treatment if resistance is conferred by a single nucleotide mutation. By combining drugs, the number of mutations necessary for complete resistance increases. In a simple scenario, whereby resistance to both drugs is conferred by a single nucleotide mutation, the chance for a single susceptible parasite to become resistant is $10^{-9} \cdot 10^{-9} = 10^{-18}$, which is much lower than the total parasite population present. The exact

genetics of resistance against the various drugs is not completely understood yet, but for most drugs it is thought that multiple mutations are necessary for a parasite to be fully resistant. Consequently, the likelihood of resistance arising against a combination of drugs can be very small (Hastings and D'Alessandro 2000; Arrow et al. 2004). Furthermore, by combining drugs with different resistance genes, resistance to both drugs can be lost following recombination due to a breakup of the two genes. Combining drugs to manage resistance is already a common practice in the treatment of tuberculosis (Blomberg and Fourie 2003) and HIV/AIDS (Yeni et al. 2002).

1.6 Why this thesis

We still do not completely understand the mechanisms behind the evolution of drug resistance (Read and Huijben 2009). Current treatment regimes are based on traditional concepts, which advise to administer high dose treatment to ensure full clearance of parasites. However, many of these treatment regimes, not only in malaria therapy, are not grounded by empirical data. For example, in antibacterial treatments, drugs are often administered longer than is necessary. Experimental analysis of patient treatment regimes to uncover whether current practices are the right ones is therefore deemed extremely necessary, since these practices lead to the proliferation of drug resistance in bacteria (Rice 2008). The same is likely true in the case of malaria.

The importance of within host dynamics to fight drug resistance has been acknowledged in the past and for a wide variety of pathogens. Over a decade ago, it was suggested that natural competitors could be used to fight drug-resistant bacteria, by 'treating' patients, infected with resistant bacteria, with susceptible wild-type bacteria (Goldhaber 1994). This controversial method of using competition to control resistance did not receive any further attention. To the best of my knowledge, the potential of harnessing susceptible pathogens in a host to control resistant ones has been mentioned only a couple more times in the literature, for instance that "*...the more effective a treatment is at eradicating drug-susceptible populations [...], the more it will promote the spread of resistant ones*" (Lipsitch and Samore 2002, pg 349) and "*...the fastest way to eliminate resistant strains is to outnumber them with susceptible strains*" (Levy and Marshall 2004, pg s125). However, empirical tests of this hypothesis are

lacking. Some evidence has come from unexpected fields of research, for instance that of cancer treatment. Cancer is, just like malaria or other infectious diseases, currently treated with a high enough treatment dose to kill as many tumour cells as possible. However, computer models for this particular disease have shown that "*if resistant populations* [of cancerous cells] *are present before administration of therapy, treatments designed to kill maximum numbers of cancer cells remove this inhibitory effect and actually promote more rapid growth of the resistant populations*" (Gatenby et al. 2009, pg 4894). A subsequent experiment using a human ovarian cancer in a mouse has shown that mice that were treated aggressively quickly had a recurrence of resistant tumour cells, while all mice that received lower dosages, although chronically infested, survived (Figure 1.8; Gatenby et al. 2009). Another set of evidence comes from an *in vitro* study using the *Staphylococcus aureus* bacteria. This experiment showed that a short course of antibacterial drugs performed better at controlling the resistant bacterial population than the conventional longer course (Figure 1.9; Drusano et al. 2009), which again argues for the use of less drugs than currently advised. Finally, the use of reduced drug pressure is implemented already in a hospital in the UK to control for antibiotic-resistant *Clostridium difficile*. Infections with *C. difficile* are the result of high antibiotic usage, which allows the resistant gut bacteria to grow to large numbers in the absence of other gut bacteria. These infections cause high mortality in hospital patients. A reduced antibiotic prescription policy was implemented, in combination with a switch to different antibiotics, which resulted in a much reduced incidence of *C. difficile* (Gulihar et al. 2009).

To my knowledge, no such work has been done on malaria parasites, or indeed any infections *in vivo* with the appropriate controls. Yet, drug resistance in malaria is one of the main hurdles in the way of malaria eradication. It is thus important to use our drugs in the most sensible way to slow down the evolution of resistance. While this thesis is based on malaria research, the logic of the arguments appears to be applicable to a wide range of diseases and will contribute to the empirical data necessary to start making changes in the conventional wisdoms that dominate treatment policies.

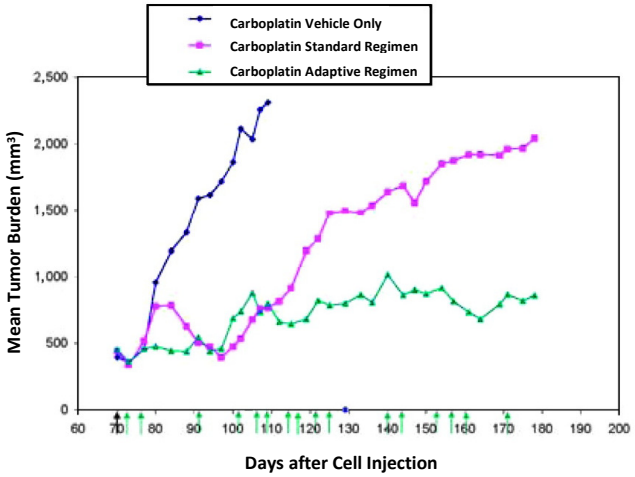


Figure 1.8 Mean tumor volume through time in mice that were inoculated with ovarian cancer cells and subsequently left untreated (blue circles), treated with a standard regimen of high dose chemotherapy (pink squares) or treated with an adaptive regimen (green triangles), which consisted of multiple low dosages of chemotherapy (indicated by the arrows) to control tumor growth. The experiment was terminated when all mice died in the untreated group and standard regimen group. All mice in the adaptive regimen group survived. Data are means for four mice in each treatment group. Details are in Gatenby et al. (2009).

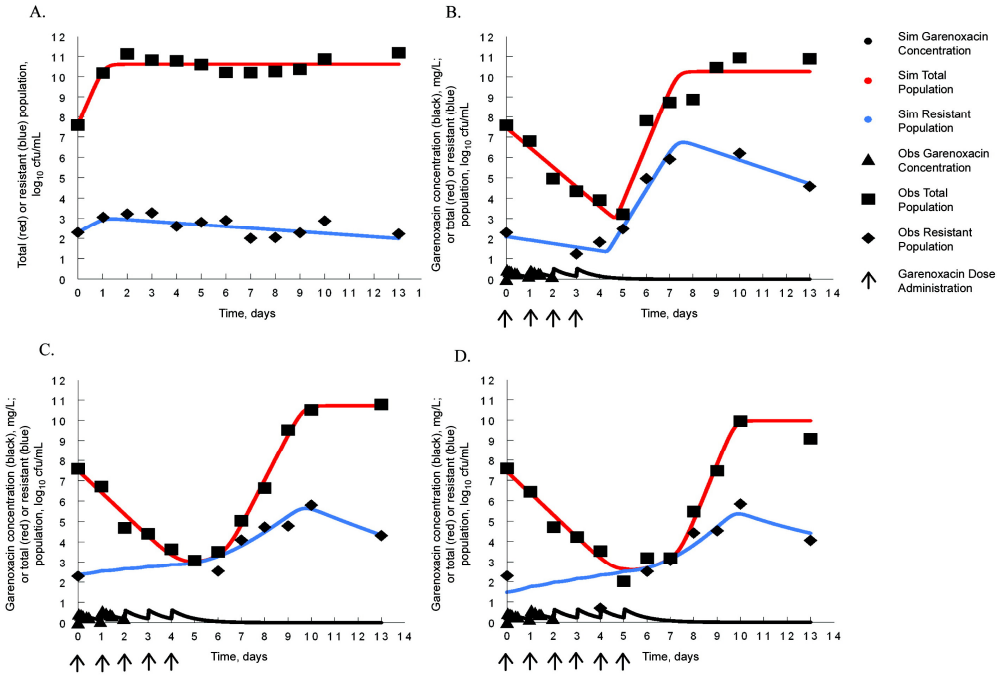


Figure 1.9 Bacterial population density in a mixture of antibacterial-resistant (blue line (simulation) and black diamonds (observed data)) and susceptible bacteria (total density is shown in red line (simulation) and black squares (observed data)) *in vitro* when receiving no antibacterial treatment (A), 4 days of treatment (B), 5 days of treatment (C) and 6 days of treatment (D). Treatment days are indicated by the arrows. The black line and triangles show the antibacterial concentration. Details are in Drusano et al. (2009).

1.7 Aims

My overall objective is to understand the within-host ecology of drug-resistant parasites, both in untreated and drug-treated infections. Understanding the within-host ecology of resistant parasites will lead to a better understanding of the evolution of drug resistance. This understanding may be a first step towards evolutionary-informed interventions, aimed at controlling the spread of drug resistance.

While one thesis is too short to fulfill the highly ambitious overall objective, in this thesis I have dealt with the following questions:

1. *What is the effect of **sub-curative drug treatment** on the within-host ecology of resistant parasites in a two-clone mixed infection?* Following up on an experiment conducted in the lab previously (Wargo et al. 2007), can reduced drug treatment reduce the extent of competitive release of resistant parasites? The rationale being that decreasing the amount of drugs given to a mixed infection of resistant and susceptible parasites leaves more susceptible parasites in the mixture to potentially suppress the resistant parasites (Chapter 2).

2. *What is the effect of the **frequency of resistant parasites** in a mixed infection on competitive suppression and release?* From previous work in the lab, we have identified strong competitive suppression on drug-resistant parasites in mixed infections of equal abundance at the outset. However, in a natural situation, resistant parasites can also occur in much lower abundances. Is competitive suppression more severe when starting out in the minority? Additionally, are these resistant parasites at low abundances being competitively released following drug treatment (Chapter 3)?

3. *Is competitive suppression and release seen in **genetically similar** drug-resistant and drug-sensitive clones?* While we have gained much knowledge on competitive suppression of resistant parasites competing with genetically distinct susceptible parasites, competition between genetically similar strains (e.g. a resistant strain and its ancestor) remains largely unstudied (Chapter 4).

4. *What is the effect of the* ***number of co-infecting genotypes*** *on the competitive suppression and release of drug-resistant parasites?* Previous experimental studies have been conducted on competition in a two-clone infection; however, field data have shown that malaria infections are frequently found to harbor more than two genotypes. Is competitive suppression more intense under these circumstances? Also, what effect does drug treatment have on resistant parasites in these multi-clone environments (Chapter 5)?

5. *Are* ***other drug treatment regimes*** *feasible that perform better at controlling drug-resistant parasites without compromising other treatment goals?* The objective of malaria drug treatment is threefold: improvement of patient health, reducing transmission intensity and control of drug resistance. The experiment described in chapter 2 showed a reduced resistant parasite density when fewer drugs were given. How does a range of different treatment regimes compare to conventional treatment based on these three treatment objectives (Chapter 6)?

1.8 Thesis arrangement

This thesis is written as a set of independent papers, with one paper representing one data chapter. Therefore, there is some inevitable repetition, particularly in the material and methods, but also in background information and the logic of arguments. To answer the above proposed questions, the *P. chabaudi* rodent malaria model was used. With this study system, I have used a specific set of parasites, drug regimes and diagnostic tools; each of these is explained in the respective chapters. The strengths and weaknesses of the rodent malaria model are discussed in the general discussion at the end of this thesis. The term 'virulence' of an infection is used throughout this thesis. Virulence is a bit of an ambiguous term; in this thesis I define virulence as the level of morbidity (in terms of anaemia and weight loss) a parasite causes to the host, which is usually strongly correlated to parasite growth rate. The drug used for these experiments is pyrimethamine. Since the motivation for the choice of this drug and background on for instance the mode of action and the molecular basis of resistance is not covered in the separate chapters, it is given below. Also, specific choices for data analysis were made. These analyses were used throughout the thesis and are therefore introduced below.

1.9 Drugs

Pyrimethamine is an antifolate drug. Malaria parasites are dependent on *de novo* folate biosynthesis. Pyrimethamine, and other folate inhibitors, exploits this dependency as a drug target. Folate is necessary for the conversion of dUMP to dTMP, which is, among other things, needed for DNA replication. Without this conversion, the cell cycle arrests and the parasite dies. One of the critical steps in the process is the conversion of dihydrofolate (H_2-folate) into tetrahydrofolate (H_4-folate) by the enzyme dihydrofolate reductase (*DHFR*, Figure 1.10). Pyrimethamine inhibits the *DHFR* enzyme by binding to the active site and thus blocking the binding of dihydrofolate, resulting in cell cycle arrest for the parasite (Foote and Cowman 1994). Therefore, following pyrimethamine treatment, all replicating parasites are killed, while the non-replicating parasites (gametocytes) are left unharmed.

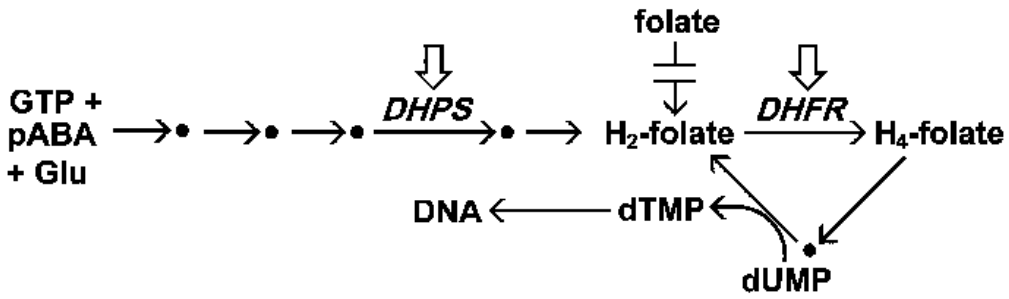


Figure 1.10 Simplified schematic version of the folate pathway. The arrow at *DHFR* shows the target site for pyrimethamine. By blocking the *DHFR* enzyme, the folate pathway arrests and the replicating parasites die (source**:**
http://www.tulane.edu/~wiser/protozoology/notes/drugs.html).

Resistance against pyrimethamine arises by a mutation in the *DHFR* gene that results in a decreased binding affinity of the enzyme to the drug (Bustamante et al. 2009). The critical mutation in *P. falciparum* is a serine to asparagine change at position 108 (Gregson and Plowe 2005), which is the same mutation observed in the pyrimethamine-resistant *P. chabaudi* clone at position 106 (Cheng and Saul 1994) used in the experiments described in this thesis. In SP-resistant *P. falciparum* in the field, more mutations are found, but the 108 mutation is always present. Most SP-resistant parasites in Africa have one or two additional mutations in the *DHFR* gene, while SP-

resistant parasites in Southeast Asia have an additional third mutation (Hyde 2008). Each additional mutation results in an increased resistance towards the drug.

Pyrimethamine-resistance is used in these studies because it is a well-established resistant parasite strain in the lab. The resistant parasites have a fully resistant phenotype: they grow just as well under drug pressure as without (e.g. de Roode et al. 2004; Wargo et al. 2007). Furthermore, the molecular basis of pyrimethamine resistance in *P. chabaudi* is largely understood. Since I am interested in the effect of drug treatment (that is, the removal of susceptible parasites from a mixed infection) on the dynamics of resistant parasites when they are already present in the population, the conclusions I obtain about this within-host ecology should qualitatively pertain to all drugs. I return to this question in the general discussion.

1.10 Data analysis

Asexual parasite densities range over 6 orders of magnitude throughout the infection; therefore, asexual parasite densities were summarized using the geometric mean over time. This approach reduces the impact on the mean density of very high counts on a few days.

Predicted infectiousness

Ideally, the outcome of within-host selection would be directly measured in terms of parasite frequencies in mosquitoes (e.g. Hallett et al. 2006). To perform this in an empirical model system requires logistically complex experiments that involve repeated mosquito feeds through time on the same mouse, or greatly increased number of mice in the study, were not conducted here. Instead, we inferred infectiousness of both clones to new hosts from the gametocyte dynamics of each clone. In earlier work, we typically used cumulative gametocyte densities over the course of infection as a measure of potential transmission (e.g. Wargo et al. 2007). However, infectiousness (proportion of mosquitoes infected) is unlikely to be a simple linear function of gametocyte density, not least because there must be a saturation effect at high gametocyte densities and less efficient transmission at low densities because of mate limitation in gametocyte reproduction (Barnes and White 2005; Paul et al. 2007; Sinden et al. 2007). Therefore, we used a density-infectivity function (or q-

function) to estimate the number of mosquitoes potentially infected with each clone of the following general form (Sinden et al. 2007):

$$q = \frac{\alpha N^{\beta}}{1 + \gamma N^{\beta}}, \qquad (3)$$

with gametocyte density denoted as N and probability of infection by q. The exact shape of the q-function is not well resolved and it may vary with epidemiological settings (Stepniewska et al. 2008). We used two different curves (Figure 1.11) both derived from experimental studies with *P. falciparum*. One comes from data compiled by Barnes and White (2005), and is based on feeding experiments on artificial *P. falciparum* infections of 88 neurosyphilitic patients using two different strains, one from South Carolina, the other from Panama (Jeffery and Eyles 1955). The other dataset comes from 6 different studies on natural *P. falciparum* infections from West Africa, summarised by Carter and Graves (1988). Ideally, we would also be able to include a curve estimated from experimental work on *P. chabaudi* (Buckling et al. 1997; Taylor et al. 1997b; Mackinnon and Read 1999). However, since most relevant data on *P. chabaudi* come from experiments designed to maximise transmission, there are too few data to estimate transmission success at gametocyte densities relevant here (post-treatment). The sensitivity of the conclusions to the choice of q-curve is considered in the general discussion.

Gametocyte abundances were used to calculate probability of infection through time using the q-curves. To translate these dynamical probabilities into a single transmission potential parameter, the number of infected mosquitoes from a potential of n=100 equally distributed across time was calculated for of each mouse. These calculations assume that there is no subsequent interaction between clones in mosquitoes which is negatively correlated with relative fitness in the vertebrate host, and that infectivity per gametocyte remains equal over the course of infection. Again, these assumptions are discussed in the general discussion.

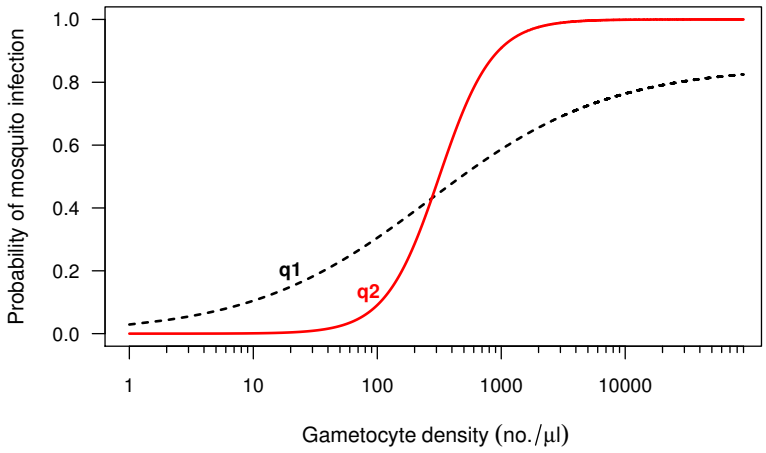


Figure 1.11 Density-infectivity q-functions used in the analysis: probability of a single mosquito becoming infectious based on gametocyte density. Function q1 (black dashed line) is based on data from Barnes and White (2005), q2 (red solid line) is based on data from Carter and Graves (1988). Probability of infection was calculated using equation (3) and parameters $\alpha = 0.03$, $\beta = 0.6$, $\alpha/\gamma = 0.85$ for q1 and $\alpha = 1 \cdot 10^{-5}$, $\beta = 2$, $\alpha/\gamma = 1$ for q2. Infectivity saturates at α/γ. Gametocyte density is plotted on a log scale.

2. Chemotherapy, within-host ecology and the relative fitness of drug-resistant malaria parasites in mixed infections

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2.1 Abstract

Drug resistance is a major problem in malaria control. The rate with which resistant parasites spread through a population is dependent on the within-host ecology of drug-resistant and drug-sensitive parasites. Drug treatment significantly shapes the within-host ecology, with drugs greatly increasing the relative fitness of resistant parasites because they have a survival advantage and an additional gain by means of reduced competitive suppression. Here we test the hypothesis that the spread of resistance can be slowed down by reducing drug treatment and thus restricting competitive release. Using the rodent malaria model *Plasmodium chabaudi*, we found that competitive release of resistant parasites was more restricted following low dose therapy compared to high dose treatment. Importantly, the lower dose improved host health to the same level as high dose treatment. In other words, high dose treatment exerted strong positive selection on resistant parasites for little clinical gain. Therefore, depending on the epidemiological context such as the frequency of mixed

infections in a particular region, our data raise the question whether the currently used, radical, high dose treatment regimes are the best resistance management strategies.

2.2 Introduction

Drug-resistance management is one of the major challenges in modern health care. Current management tools are primarily targeted at reducing the likelihood of *de novo* resistant mutants to arise in a treated patient (Arrow et al. 2004; WHO 2006). This may be a sensible approach for diseases with highly mutable pathogens such as the HIV/AIDS virus. Yet, in diseases where widespread drug resistance is predominantly caused by spread of resistant strains as opposed to frequently arising *de novo* mutants such as appear to be the case in malaria (Cortese et al. 2002; Wootton et al. 2002; Nair et al. 2003; Hastings 2004; Roper et al. 2004) and perhaps also tuberculosis (Luciani et al. 2009), other resistance management tools may be more effective. In this latter case, the useful lifespan of a drug, which is the time from introduction of a new drug to a treatment failure rate exceeding 10% (WHO 2006), is dependent on the strength of selection for drug-resistant parasite strains (Hastings 2003). The strength of selection is a function of the relative fitness among resistant and susceptible parasites, and depends among other things on the within-host ecology of drug-resistant and drug-sensitive parasites. The within-host ecology of resistant and sensitive parasites is affected by a multitude of factors, most importantly drug pressure.

High level drug resistance has evolved against most classes of first-line antimalarial drugs, making the evolution of drug resistance a major obstacle in malaria control (Greenwood et al. 2008). Two widely used drugs, chloroquine and sulphadoxine-pyrimethamine (SP), have become useless in most parts of the world (Hyde 2005). Interestingly, the vast majority of strains resistant to chloroquine and SP that are currently circulating in Africa have not arisen in Africa itself, but instead originated from resistant strains from Southeast Asia (Cortese et al. 2002; Wootton et al. 2002; Nair et al. 2003; Roper et al. 2003; Hastings 2004), although there is some discussion that a few highly resistant SP strains may have their origin in Africa (Mita et al. 2009). In any event, repeated *de novo* origin of resistant mutants appears to be rare, which means that most people who harbour a drug-resistant infection have received these

resistant parasites from someone else. Hence, by controlling the spread of resistant parasites, the useful lifespan of the drug could be increased.

Multiple genotype infections are common in malaria patients (e.g. Arnot 1998; Babiker et al. 1999; Smith et al. 1999; Bruce et al. 2000; Jafari et al. 2004; A-Elbasit et al. 2007; Nwakanma et al. 2008; Vafa et al. 2008; Baruah et al. 2009; Soulama et al. 2009). This means, especially when resistance is rare, that co-infections of resistant and susceptible parasites are very common. When mixed infections of drug-resistant and drug-sensitive parasites are drug-treated, the relative fitness of resistant parasites increases simply because of a survival advantage resistant parasites have over drug-sensitive ones. Additionally, diminished competitive suppression by the susceptible parasites following drug treatment has been found to increase the relative frequency of the resistant parasites even further (Hastings 2003; de Roode et al. 2004; Wargo et al. 2007). This competitive release allows the resistant parasites to fill up the ecological space left by susceptible parasites. Direct experimental evidence on competition between co-infecting *P. falciparum* genotypes, particularly with drug-resistant parasites, cannot be ethically obtained from human infections. However, field data showing suppressed population densities of a genotype when other genotypes are present strongly supports the presence of between-genotype competition within malaria-infected hosts (Daubersies et al. 1996; Mercereau-Puijalon 1996; Smith et al. 1999; Bruce et al. 2000; Hastings 2003; Talisuna et al. 2006; Bousema et al. 2008; Harrington et al. 2009). Additionally, there is substantial experimental evidence for crowding in rodent malaria models (e.g. Jarra and Brown 1985; Taylor et al. 1997; de Roode et al. 2004; de Roode et al. 2005b; Bell et al. 2006; Wargo et al. 2007). Competitive suppression and competitive release have also been demonstrated in mixed infections of drug-resistant and drug-sensitive *P. chabaudi* parasites. Drug-resistant parasites were competitively suppressed in the absence of drug treatment and competitive release was subsequently seen following prophylactic (de Roode et al. 2004) and therapeutic (Wargo et al. 2007) drug treatment. Additionally, competitive facilitation was observed, whereby resistant parasites had higher parasite abundance than in the absence of competition (Wargo et al. 2007).

This theory suggests that the spread of drug resistance can be slowed by any drug treatment that limits competitive release (Wargo et al. 2007). The logic is as follows.

Current drug treatment is aimed at eliminating all susceptible parasites from the infection. Less aggressive treatment, involving for instance shorter the treatment courses or lower drug doses, would result in a partial clearance of susceptible parasites, leaving these remaining susceptible parasites to, at least partially, suppress the co-infecting resistant parasites and hence reduce competitive release of resistant parasites. This hypothesis was tested by treating acute infections with either one, two or four days of drug treatment (Wargo et al. 2007). Both incomplete drug regimes of one or two days did not fully clear the infection of drug-sensitive parasites, however, all treatments still resulted in competitive release of the resistant parasites. The release of resistant parasites was significantly reduced in the one day treatment group compared to longer treatment durations.

Here we test the hypothesis that the extent of competitive release (absolute fitness gain for resistant parasites) can be restricted still further by even lower or shorter regimens while still alleviating the clinical symptoms of disease. We were interested to see the fitness outcome for drug-resistant genotypes over the full course of infection and thus monitored infections for a longer duration than our previous studies. Greater sensitivity in malaria transmission stage (gametocyte) quantification and higher infected host replication enabled us to quantify more accurately than was previously possible the variable and low level dynamics that are known to occur in late stage infections. We also introduce two new analytical methods. First, to more precisely describe relative transmission success of resistant parasites, we made use of functions relating gametocyte density to infectivity (e.g. Stepniewska et al. 2008). Second, to better define the evolutionary consequences of drug treatment, we estimated the duration and strength of selection through the course of infection.

We found that competitive release occurred after a single high drug dose. Resistant parasites were also released following low dose treatment, but the extent of release was more restricted. Host health was improved to the same level for both treatments, so that high dose treatment led to stronger positive selection on resistant parasites for little clinical gain.

2.3 Material and methods

2.3.1 Parasites and hosts

Two genetically distinct *P. chabaudi* clonal lineages were used to inoculate the hosts: drug-resistant $AS_{12265(pyr\text{-}1A)}$ (hereafter referred to as clone R) and drug-sensitive AJ_{5154} (hereafter referred to as clone S). Clone R was made resistant to pyrimethamine after isolation by a single passage with high dose treatment (Walliker et al. 1975) and has been exposed to pyrimethamine in two later passages as well. Clone S has not been exposed to pyrimethamine since isolation (Beale et al. 1978). Hosts were six to eight week old female C57Bl/6J mice (Harlan, UK) maintained on a 41B maintenance diet (Harlan, UK), with their drinking water supplemented with 0.05% para-amino benzoic acid (PABA) to enhance parasite growth (Jacobs 1964).

2.3.2 Experimental design and infections

Mice were inoculated with one or both clones, with an intra-peritoneal injection of 10^6 parasites of each clone. In mixed infections, two separate inoculations were given so that mixed infections received twice the parasite dose of single-clone infections. We did this because analysing competition requires comparison of the performance of an individual clone in the presence and absence of competition, starting from the same initial parasite dose. A two fold dose of 10^6 parasites has a negligible effect on parasite dynamics or host health (Timms et al. 2001). Drug treatment was given on day 6 post-infection (PI), when parasite induced weight loss and anaemia became pronounced. Drug treatment consisted of either 8 mg pyrimethamine/kg bodyweight (high dose), 3 mg/kg body weight (low dose), or no drugs (negative control). Note that both these drug treatments were expected not to fully clear the susceptible parasites and fall well short of 8 mg/kg pyrimethamine for four successive days, which is the standard drug treatment regimen in this model system to fully clear susceptible parasites (e.g. de Roode et al. 2004; Wargo et al. 2007). Pyrimethamine was dissolved in dimethyl sulfoxide (DMSO) and an inoculum of 50 µl was given intra-peritoneal to each mouse. Untreated mice were inoculated with 50 µl of DMSO only. Each treatment group consisted of six mice, totalling to 54 mice (Table 2.1).

Table 2.1 Experimental set-up of the study. Each treatment group consisted of six mice at the start of the experiment.

	No drugs	Low dose	High dose
Single-clone infection R	6	6†*	6
Single-clone infection S	6†††	6	6
Mixed infection R+S	6	6	6*

† denotes a dead or euthanized mouse, * represents a mouse excluded because of a substantially low parasite dose.

2.3.3 Monitoring of infections

Mice were monitored daily from day three to 21 PI and thereafter on days 24, 26 and 28 PI. Host health was monitored by measuring mouse body mass (to the nearest 0.01 gram) and red blood cell density using flow-cytometry (Beckman Coulter) of a 2 µl sample of blood from a tail snip (Taylor et al. 1998; Mackinnon et al. 2002a). Quantitative real-time PCR was used to determine asexual parasite and gametocyte densities for each clone using clone-specific assays, for which an additional 5 µl and 10 µl of tail snip blood was taken respectively.

DNA was extracted from 5 µl blood using the BloodPrep kit (Applied Biosystems, Foster City, CA) on the ABI Prism 6100 Nucleic Acid PrepStation according to manufacturer's instructions. DNA was eluted in a total volume of 200 µl and stored at -80°C until quantification (Bell et al. 2006). RNA was extracted from 10 µl of blood, using the 'RNA Blood-DNA' method (Applied Biosystems) on the ABI Prism®6100 Nucleic Acid PrepStation, following the manufacturer's protocol, and eluted in 100 µl elution solution. Following RNA extraction, single stranded cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems) and stored at -80°C until quantification (Wargo et al. 2006; Wargo et al. 2007).

PCR was performed on DNA to quantify the respective total parasite densities, and subsequently also on cDNA-converted RNA to quantify the number of gametocytes (Drew and Reece 2007). Asexual parasite densities were calculated by subtracting gametocyte counts from total parasite counts. The PCR reaction was identical for both clone-specific assays. Each reaction, with a final volume of 25 µl, consisted of 7 µl of DNA or cDNA, 900 nM forward and reverse primers, 250 nM probe and 1X concentration TaqMan® Universal PCR Master Mix (Applied Biosystems). All reactions

were run on the ABI Prism® 7000 Sequence Detection System using the assay: 50°C for 2 minutes, 95°C for 10 minutes, then 45 cycles of: 95°C for 15 seconds and 60°C for 1 min. For primer and probe sequences, see Drew and Reece (2007). Quantification was based on serial dilutions of DNA and cDNA standards of known parasite and gametocyte density respectively, determined beforehand by microscopy.

2.3.4 Data analysis

Selection coefficients

To estimate the temporal pattern of the strength and direction of within-host selection, the coefficient of selection on the resistant clone was estimated. In a two-clone infection, the selection coefficient is the difference in the per-capita growth rate of each clone. This strength of selection, denoted by $s_i(t)$, can be calculated from the frequency of the competitors:

$$s_i(t) = \frac{dp_i(t)}{dt}\frac{1}{p_i(t)(1-p_i(t))}, \qquad (1)$$

where $p_i(t)$ is the relative abundance of competitor *i* at time *t* (Nelson et al. 2005). Essentially, selection reflects how quickly competitor proportions are changing, modified by how close they are to loss or fixation. The approach is to fit a time-series model to the parasite dynamics, which allows a statistical representation of the dynamics in relative abundance and, by using equation (1), a statistical description of the selection dynamics. A time-series model was fitted to the asexual parasite and gametocyte dynamics for each clone in each mouse. This resulted in four time-series models from each mouse, and two estimates of selection dynamics—one for selection between the strains in the asexual stage, and one for selection between strains in the gametocyte stage. The time-series model was a Generalized Additive Model (GAM) with a quasi-likelihood distribution (McCullagh 1983).

$$y_i(t) = s_i(t)$$
$$Y_i(t) \sim Q(y_i(t), V(y))$$

where $s(t)$ is a smoothing cubic spline that will represent the fit parasite dynamics. The GAM is setup with the standard modified objective function that includes a likelihood term that measures how well the model fits the data, as well as a wiggliness term that adds a penalty for excessive curvature (Wood 2006). The tradeoff parameter for the modified objective function was estimated generalized cross validation. The expected parasite dynamics are denoted by $y(t)$, and the observed parasite dynamics by $Y(t)$.

The observed parasite dynamics are distributed as $Q(y(t),V(y))$, which has a quasi-likelihood distribution with an expectation $y(t)$ and a mean-variance relationship denoted by $V(y)$.

Since parasite dynamics span twelve orders of magnitude in natural log units (6 in $\log_{10}$ units), we need to pay particular attention to the mean-variance function $V(y)$. To estimate this relationship, we conducted a series of additional mouse trials where replicate samples were taken on days 3-15 PI from mice infected with clone R (see appendix to this chapter). These data suggest a power relationship between the mean (y) and variance ($V(y)$) of both asexual parasite and gametocyte densities. The function has the form

$$V(y) = ay^b \qquad (2)$$

which is the general form for Tweedie distributions where a is the dispersion parameter, and b determines the specific class of distribution (Jorgensen 1987). For asexual parasites, b = 1.59, which falls within the range of compound Poisson distributions. For gametocytes, b = 2.206, which falls into the Gamma distribution class. Non-integer values of b>2 yield compound distributions that are difficult to implement in a GAM framework. To accommodate the implementation, we assumed b=2 for both asexual and gametocyte distributions, which still provides a good fit to the raw data (see appendix to this chapter). With b=2, the dispersion parameter (a) was refit, resulting in a = 0.01415 for asexuals and a = 0.0582 for gametocytes. For all fits, we used this observed value of dispersion rather than estimating it from the time-series data during the fitting process.

Parametric bootstrapping was used to estimate the confidence bands around the estimated selection dynamics for the asexual parasites and gametocytes in each mouse. The upper and lower 95% confidence bands for selection dynamics were calculated from 5000 bootstraps replicates.

Statistical analysis

Analyses were performed in R 2.9.0 (R Development Core Team 2009). Host health was summarized as arithmetic mean mouse body mass and arithmetic mean red blood cell density, with minimum red blood cell density and minimum body mass taken as a measurement of morbidity during the acute infection. The arithmetic mean of these

virulence measures and the geometric mean asexual parasite density were calculated for each mouse over the post-treatment infection period (day 7-28 PI). To ensure treatment groups with the same clonal infections did not differ prior to drug treatment, day 6 values on asexual parasite density, body mass and red blood cell density were evaluated for statistical differences. No statistical differences were found. The predicted infectiousness was calculated on the post-treatment (day 7-28 PI) gametocyte abundances as described in the general introduction.

General linear modeling was used with the following factors: *competition* (single/mixed) and *drugs* (no drugs/low dose/high dose). Maximal models with the interaction terms were fitted first and subsequently removed if not significant. When comparing the effect of drugs on single or mixed infections only, a one-way ANOVA was performed. To test for differences among drug treatment groups, Tukey HSD (Honestly Significant Difference) post-hoc multiple comparison tests were carried out and the adjusted p-values are reported. The analysis on the predicted number of infected mosquitoes was done separately for single and mixed infections using factors *drugs* (no drugs/low dose/high dose) and *clone* (R/S). Gametocyte densities did differ significantly between treatment groups with the same clonal infections prior to treatment on day 6, therefore, day 6 gametocyte densities were included in the model as a covariate.

Six mice were excluded from the analysis (Table 2.1). Four mice died or were euthanized during the acute phase of the infection and two evidently received a substantially lower parasite dose (parasite density at time of treatment two orders of magnitude lower than expected; Table 2.1). Note that we deliberately chose a mouse strain, sex and size which we know minimizes risk of death; the infection kinetics of both clones in the four mice that did die were qualitatively identical to those in mice that did not die.

2.4 Results

2.4.1 Asexual parasite dynamics

As expected from previous studies (de Roode et al. 2004; Wargo et al. 2007), parasites from clone R and S were indeed respectively resistant and susceptible to treatment with pyrimethamine. In single clone infections, the densities of asexual parasites of clone R were unaffected by drug treatment (Figure 2.1a, *drugs*: $F_{2,13}$=0.2, p=0.78), whereas clone S densities were reduced (Figure 2.1b, *drugs*: $F_{2,12}$=26, p<0.001). The high drug dose killed more S parasites than the low drug dose (Single-clone infections - Tukey HSD, *high dose vs. low dose*: p_{adj}<0001). The low drug dose resulted in an initial kill of S parasites, but over the period following drug treatment, an equal amount of S parasites were produced compared to untreated infections (Tukey HSD, *low dose vs. no treatment*: p_{adj}=0.85).

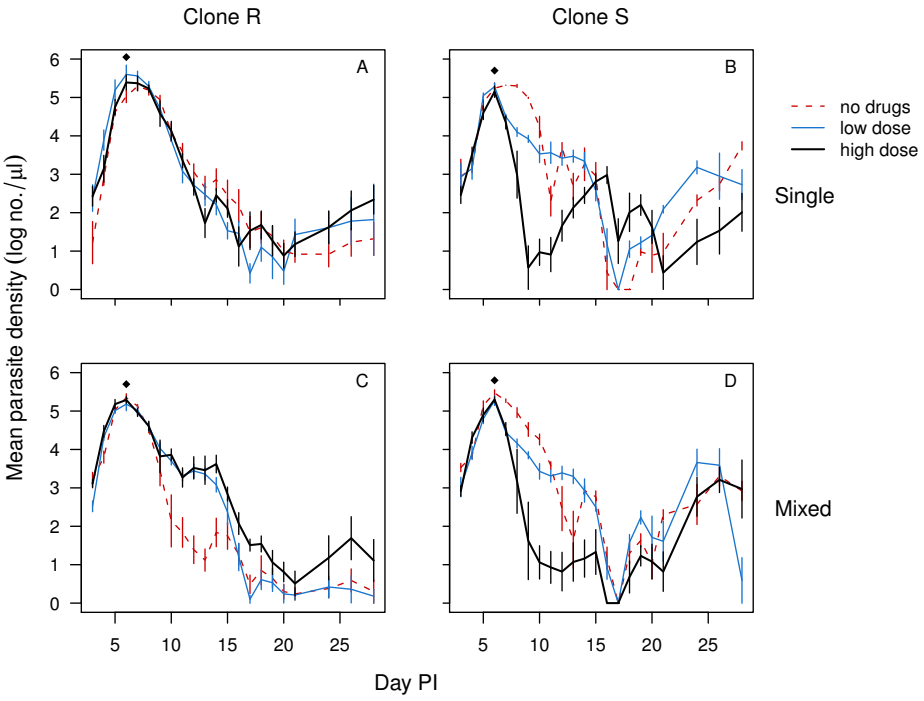

Figure 2.1 Parasite densities of the drug-resistant clone R (left panels) and the drug-sensitive clone S (right panels) in single (top panels) and mixed (bottom panels) infections that were either left untreated (dashed red line), received a low drug dose (solid blue line) or high dose (solid black line). Black diamonds indicate the timing of drug-treatment at day 6 post-infection. Data are geometric means (± standard error) for up to six mice (Table 2.1).

In untreated mixed infections, densities of clones R and S increased similarly to peak parasitaemia, after which clone R parasite densities declined more rapidly than parasite densities of clone S (Figure 2.2, left column). This was due to competitive suppression, whereby fewer R parasites were produced when susceptible parasites were present (Figure 2.1c, *competition*: $F_{1,27}=15.6$, $p<0.001$). The performance of clone S was unaffected by the presence of clone R, with as many susceptible parasites persisting for as long in single and mixed infections in the absence of drug treatment (Figure 2.1d, *competition*: $F_{1,28}<0.1$, $p=0.94$).

As in single-clone infections, densities of clone S parasites in mixed infections were greatly reduced by drug treatment. The two drug dosages had similar rates initially, but after two days the decline of S parasites was greater following high dose treatment (Figure 2.1bd, 2.2).

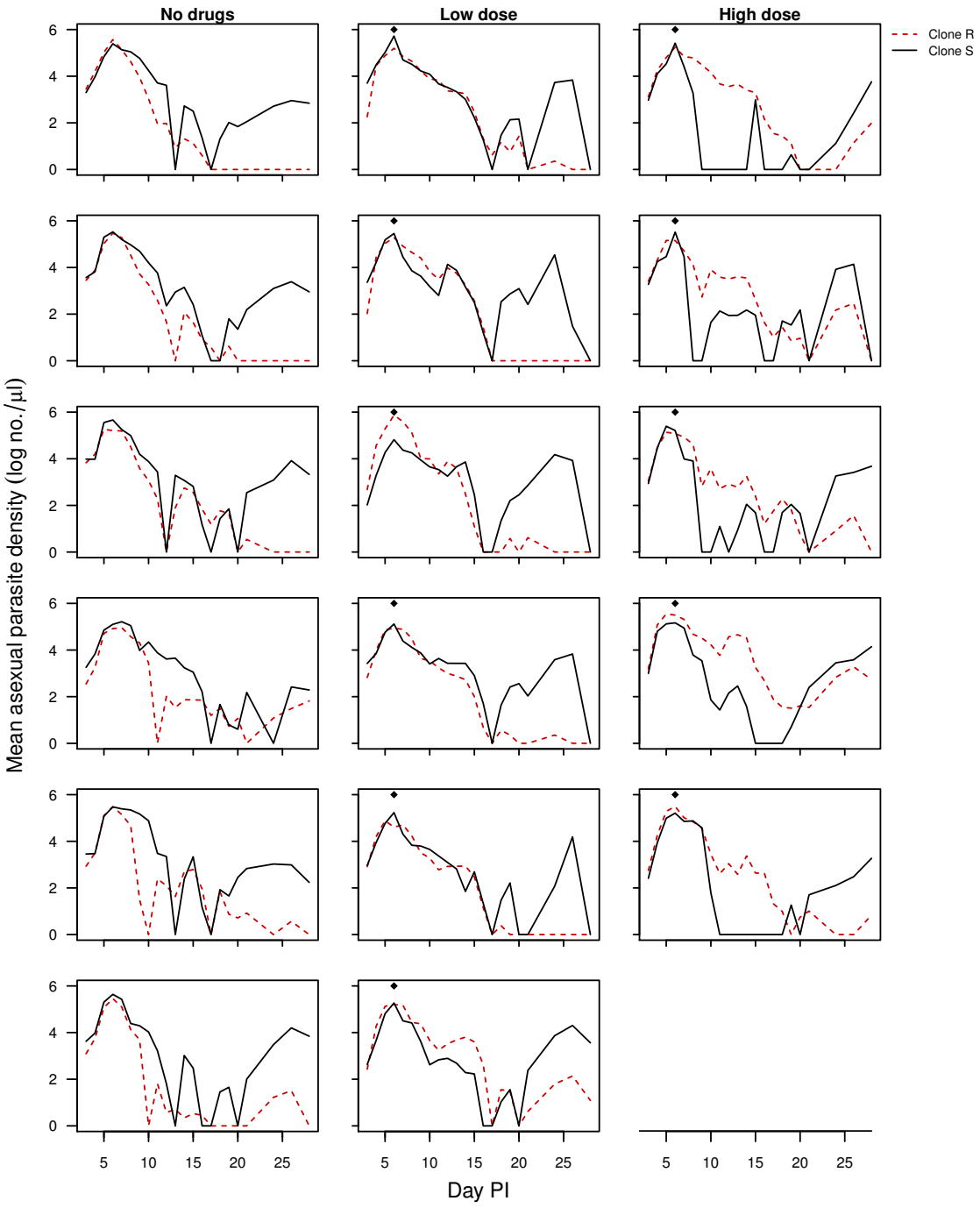

Figure 2.2 Asexual parasite dynamics of individual mice that were infected with a mixed infection of clone R (dashed red line) and clone S (solid black line), which received no drug treatment (left column), a low dose of pyrimethamine (middle column) or a high dose of pyrimethamine (right column). Each group consisted of 6 mice at the outset of the experiment; however, one mouse in the high dose treatment group received a much lower parasite dose than intended and was excluded (Table 2.1). Drug treatment was given on day 6 post-infection, as indicated by the black diamonds.

Three days after treatment, there were as many or more resistant parasites in drug-treated mixed infections than in single-clone infections (competitive facilitation; Figure 2.1c, 2.2, 2.3a, *competition x drugs*: $F_{2,27}$=5.8, p=0.008). The higher drug dose resulted in more than half as many resistant parasites post-treatment than in untreated infections and almost a quarter more than low dose treatment (mixed infections - Tukey HSD, *no drugs vs. high dose*: p_{adj} =0.002, *low dose vs. high dose*: p_{adj}=0.098).

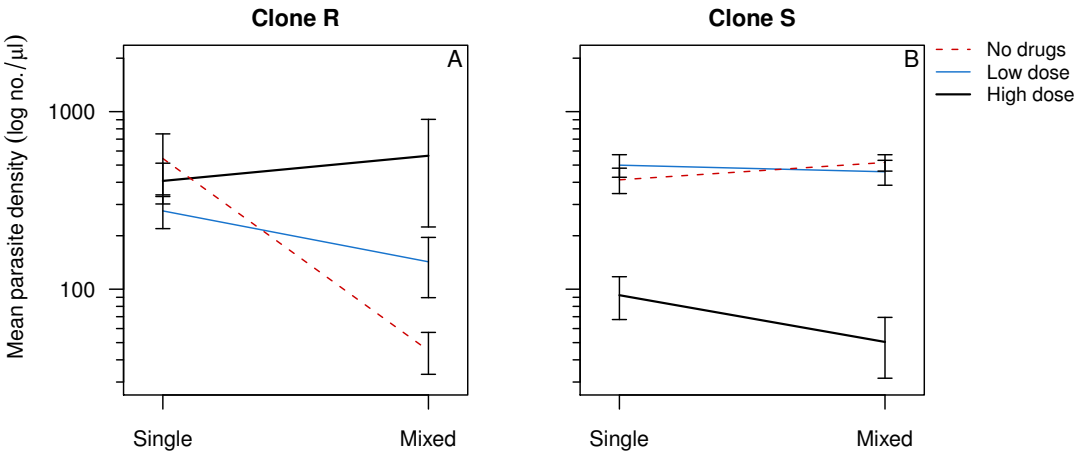

Figure 2.3 Geometric mean asexual parasite density (± standard error) of clone R (left plot) and clone S (right plot) in single and mixed infections that were either left untreated (dashed red line), received a low drug dose (solid blue line) or high dose (solid black line). Data are the arithmetic means of the geometric mean density per day over the course of post-treatment infection for up to six mice (Table 2.1).

Together, these data show that densities of resistant parasite were suppressed when drug-sensitive parasites were present. Removing those sensitive parasites with drug treatment led to competitive release, and subsequently greatly increased densities of resistant parasites compared to untreated mixed infections.

2.4.2 Transmission potential

Gametocytes of both clone R and S peaked on or around day 6 and subsequently again around day 13 in untreated single-clone infections (Figure 2.4, 2.5). Clone S peaked at higher gametocyte densities than clone R and typically had an additional gametocyte

peak after day 20 post-infection (Figure 2.4ab). As with the asexual dynamics, gametocyte densities for clone R were, in the absence of drug treatment, suppressed when sensitive parasites were present (Figure 2.4c), while clone S achieved similar gametocyte densities in untreated single-clone and mixed infections (Figure 2.4d). The day after high dose drug treatment, clone S had higher gametocyte densities, both in single and mixed infections (Figure 2.4bd). On subsequent days however, gametocyte densities from the sensitive clone were dramatically reduced. Following low dose treatment, gametocyte densities a day later were not elevated. Clone S in mixed infections did not produce the second gametocyte peak around day 13 following high dose treatment but they did following a low dose. All mixed infections demonstrated a third S gametocyte peak (Figure 2.4d, 2.5). In line with the competitive release of asexual densities following chemotherapy, resistant gametocyte densities in mixed infections were also elevated, particularly following high dose treatment (Figure 2.4c, 2.5).

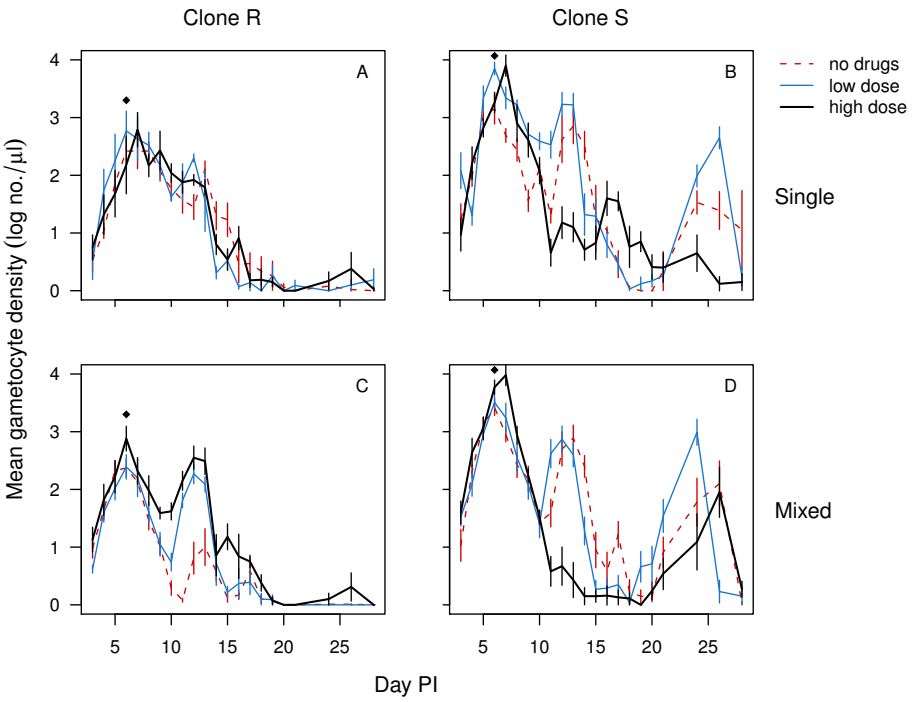

Figure 2.4 Gametocyte densities of the drug-resistant clone R (left panels) and the drug-sensitive clone S (right panels) in single (top panels) and mixed (bottom panels) infections that were either left untreated (dashed red line), received a low drug dose (solid blue line) or high dose (solid black line). Black diamonds indicate the timing of drug-treatment at day 6 post-infection. Data are geometric means (± standard error) for up to six mice (Table 2.1).

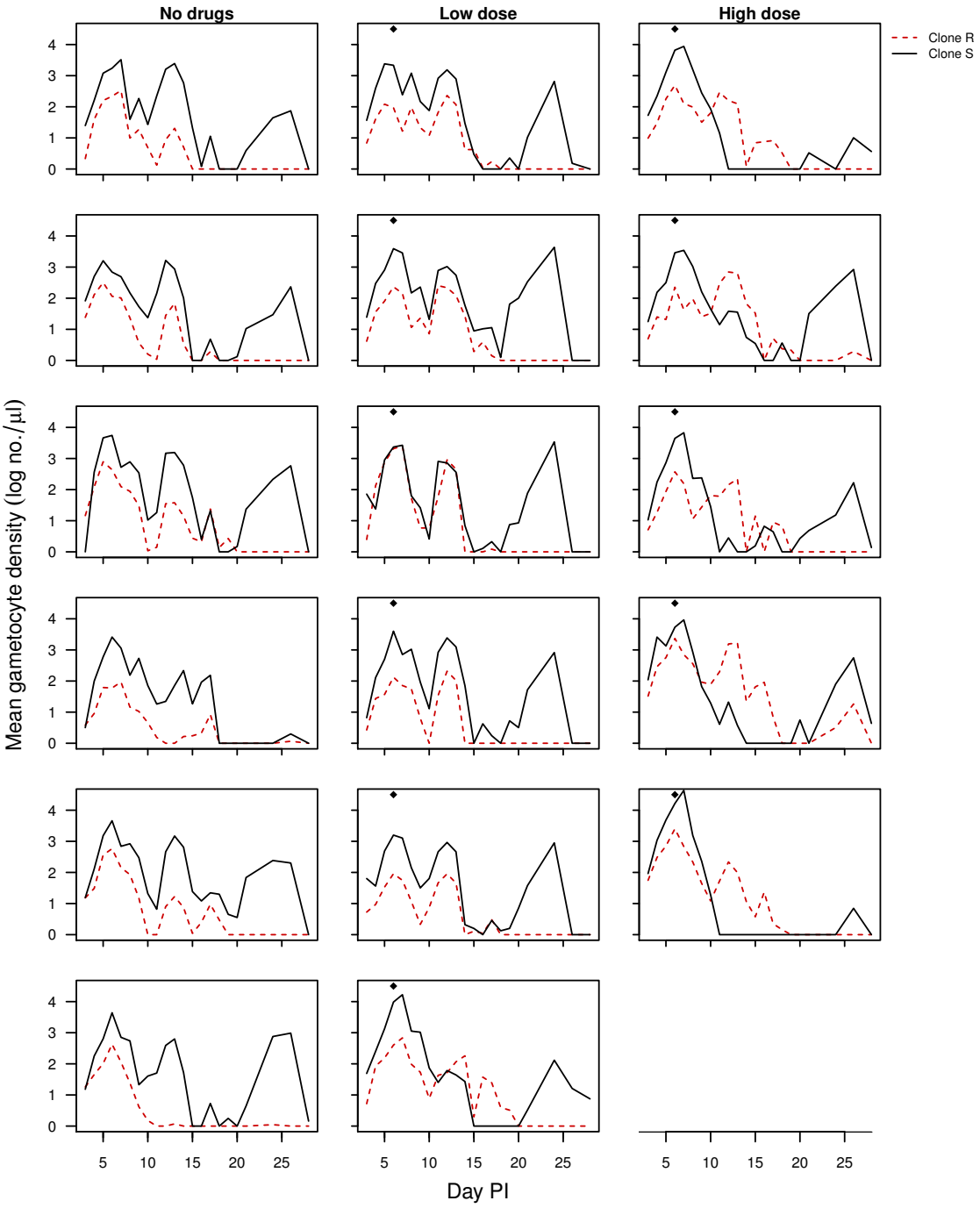

Figure 2.5 Gametocyte dynamics of individual mice that were infected with a mixed infection of clone R (dashed red line) and clone S (solid black line), which were either left untreated (left column), received a low drug dose (middle column) or high dose (right column). Each group consisted of 6 mice at the outset of the experiment; however, one mouse in the high dose treatment group received a much lower parasite dose than intended and was excluded (Table 2.1). Drug treatment was given on day 6 post-infection, as indicated by the black diamonds.

With high drug dose, the number of mosquitoes predicted to be infected from mixed infections was increased for R parasites and decreased for S parasites (Figure 2.6a; Table 2.2, *drug* x *dose* interaction). Using the q2 function based on data from Barns and White (2005), high dose drug treatment converted a more than three-fold advantage for clone S in untreated infections into parity (Figure 2.6a), thus losing the advantage clone S had over clone R. In single-clone infections, drug treatment did not affect predicted infectivity of clone R, but it did for clone S (Figure 2.6b; Table 2.2, *drug* x *dose* interaction). Surprisingly, a clear downward trend for gametocyte densities of clone S was not observed, which is a result of elevated gametocyte densities the day after high dose treatment and elevated second gametocyte peaks in low dose treatment (Figure 2.4b).

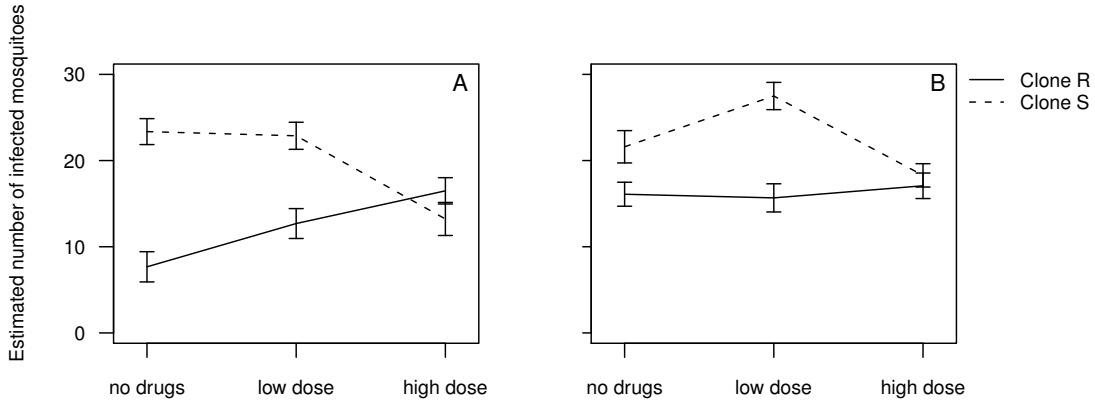

Figure 2.6 Least square mean (± standard error) predicted number of infected mosquitoes (out of n=100) with clone R (solid line) and clone S (dashed line) from mixed (a) and single (b) infections that were either left untreated, received a low drug dose or high drug dose. Predicted infectivity is based on the post-treatment gametocyte densities using density-infectivity function q2 (Figure 1.11); a similar picture was seen using function q3. Least square means and standard errors were calculated from the statistical model whereby covariate gametocyte density on day 6 was kept constant.

Together, these data show that the relative fitness of resistant parasites in mixed infections was increased by drug treatment in two ways. First, susceptible parasite fitness decreased, particularly at the high dose treatment. Second, the absolute fitness of resistant parasites increased as a consequence of competitive release (Figure 2.6a). In single-clone infections, relative fitness of resistant parasites was only increased by the survival advantage, and this was only observed in the high dose treatment (Figure 2.6b). The transmission potential of the resistant parasites never exceeded that of the

susceptible parasites, which was the aim of the drug treatments chosen in this experiment.

Table 2.2 Analysis of variance table of mixed and single-clone infections to test for an effect of drug treatment (*drugs*: no drugs/low dose/high dose), parasite clone (*clone*: R/S) and the interaction between them, on predicted number of infected mosquitoes based on two density-infectivity functions (Figure 1.11; q1 and q2) using the post-treatment gametocyte densities (day 7-28). Gametocyte densities on day 6 were included as a covariate.

	q1	q2
mixed infections		
drugs	$F_{2,27}$=1.7, p=0.20	$F_{2,27}$=1.8, p=0.19
clone	$F_{1,27}$=90, p<0.001	$F_{1,27}$=100,p<0.001
drugs x clone	$F_{2,27}$=22, p<0.001	$F_{2,27}$=11, p<0.001
day 6 gametocytes	$F_{1,27}$=5.6, p=0.025	$F_{1,27}$=8.1, p=0.008
single-clone infections		
drugs	$F_{2,24}$=14, p<0.001	$F_{2,24}$=28, p<0.001
clone	$F_{1,24}$=47, p<0.001	$F_{1,24}$=53, p<0.001
drugs x clone	$F_{2,24}$=7.2, p=0.004	$F_{2,24}$=9.3, p=0.001
day 6 gametocytes	$F_{1,24}$=7.1, p=0.014	$F_{1,24}$=11, p=0.002

2.4.3 *Total parasite dynamics*

The total parasite burden (clone R + clone S) of the mixed infections was unaffected by drug treatment (Figure 2.7a, *drugs*: $F_{2,14}$=0.1, p=0.90). This is because the susceptible parasites, which dominate in the absence of treatment, are replaced by resistant parasites if treatment is given. Similar dynamics were seen for total gametocyte densities in mixed infections (Figure 2.7b). Drug treatment also did not affect overall predicted infectiousness, again because resistant gametocytes dominate in drug-treated infections while susceptible gametocyte dominate in untreated infections (*drugs*, q1: $F_{2,13}$=1.3, p=0.30; q2: $F_{2,13}$=1.6, p=0.23).

2.4.4 Host health

Unsurprisingly, drug treatment of infections consisting of only resistant parasites did not improve host health, since clone R was resistant to treatment (Figure 2.8ab, *drugs*-maximum RBC loss: $F_{2,13}$=0.2, p=0.84; maximum weight loss: $F_{2,13}$=0.1, p=0.90). In contrast, drug treatment alleviated morbidity in all infections containing S parasites (Figure 2.8c-f, *drugs*: maximum RBC loss: $F_{2,29}$=15, p<0.001; maximum weight loss: $F_{2,29}$=2.9, p=0.073). Following drug treatment, mice with mixed infections were less anaemic than untreated infections (*drugs*: mean RBC density: $F_{2,14}$=6.9, p=0.008).

Importantly, this was independent of drug dose (Tukey HSD, *low dose vs. high dose*-mean RBC density: $p_{adj}=0.46$), so that mice given the low dose treatment were no more anaemic than those given the high dose. Drug treatment had no impact on mean body mass of mice (*drugs*: mean body mass: $F_{2,14}=0.7$, $p=0.53$).

2.4.5 Selection dynamics

Drug treatment had a pronounced effect on the kinetics of selection on drug-resistant parasites throughout the infection. The rate of selection on clone R through time for each mouse is shown in figure 2.9a-c (selection on asexual parasite densities) and figure 2.9d-f (selection on gametocyte densities).

In the absence of treatment, the resistant parasites in 5 out of 6 mice were under negative selection on asexual parasite densities from approximately day 7 to day 12, after which selection became positive in half of the mice. When infections were treated with a single high dose of pyrimethamine, the opposite occurred: resistant parasites were under positive selection for the first period following drug treatment, and negative selection later on. Under low dose treatment, there was no indication of positive selection, with the exception of a few short periods in two mice. Later on in the infection, resistant parasites in this treatment group were also selected against. Positive selection of resistant gametocytes was very strong in all infections that received a high drug dose. In untreated and low dose treatment infections, little positive or negative selection on R gametocytes was observed.

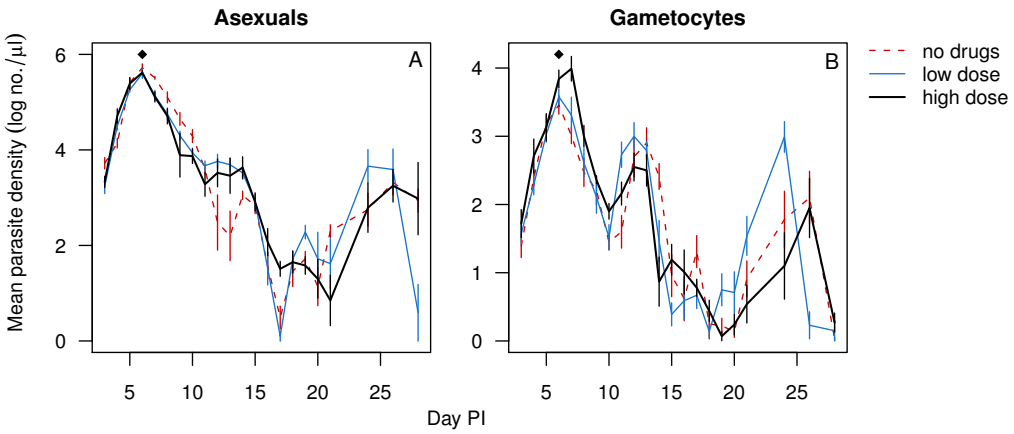

Figure 2.7 Total asexual parasite (left graph) and gametocyte (right graph) dynamics of mixed infections that were either left untreated (dashed red line), received a low drug dose (solid blue line) or high dose (solid black line). Black diamonds indicate the timing of drug-treatment at day 6 post-infection. Data are geometric means (± standard error) for up to six mice (Table 2.1).

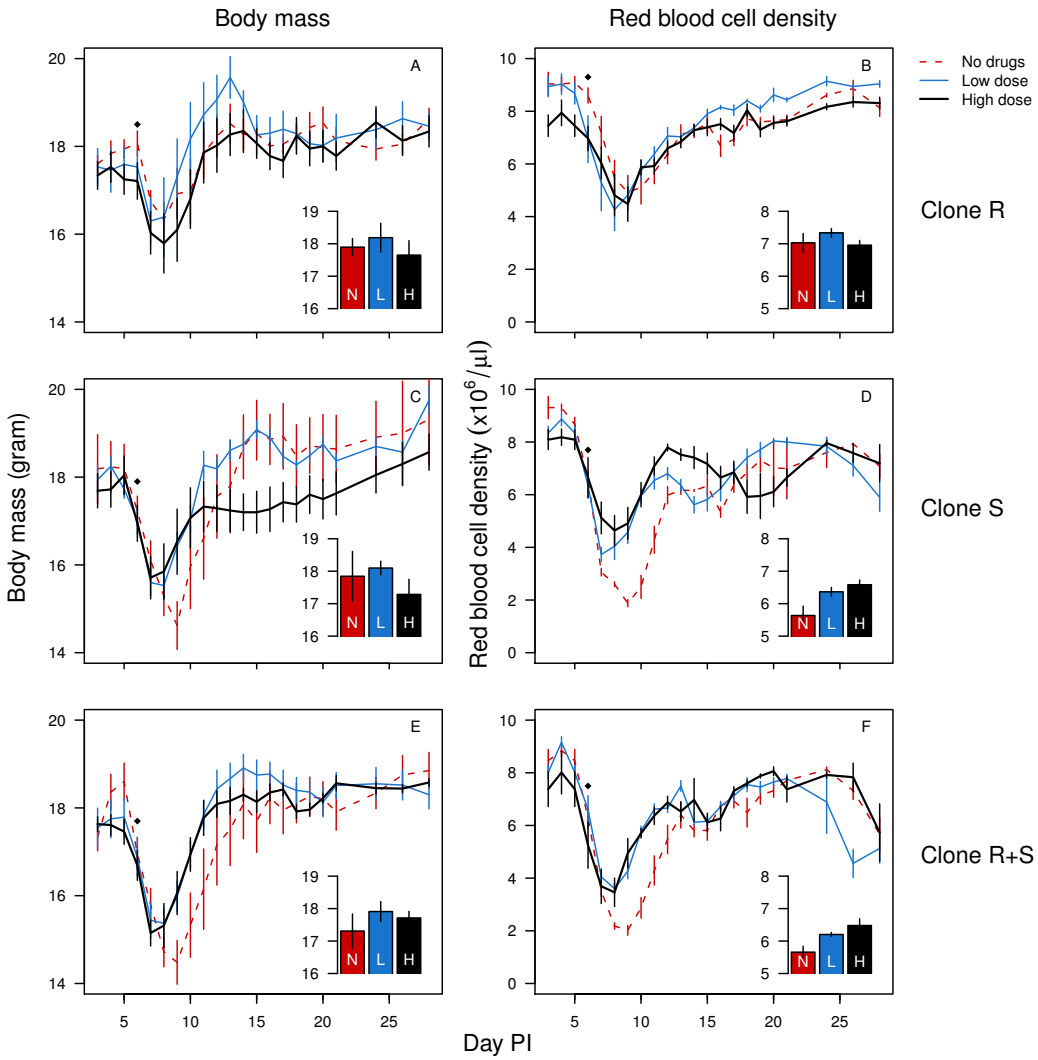

Figure 2.8 Body mass (± standard error, left panels) and red blood cell density (± standard error, right panels) of mice infected with drug-resistant clone R (top panels), drug-sensitive clone S (middle panels) and mixed infections of both clones (bottom panels) for untreated infections (dashed red line), low dose treatment (solid blue line) and high dose treatment (solid black line). Black diamonds indicate timing of drug treatment at day 6 post-infection. The inset bar charts show the mean body mass and mean red blood cell density post-treatment (± standard error) for untreated infections (red bars - "N"), low dose treatments (blue bars - "L") and high dose treatments (black bars – "H"). All data are arithmetic means for up to six mice (Table 2.1).

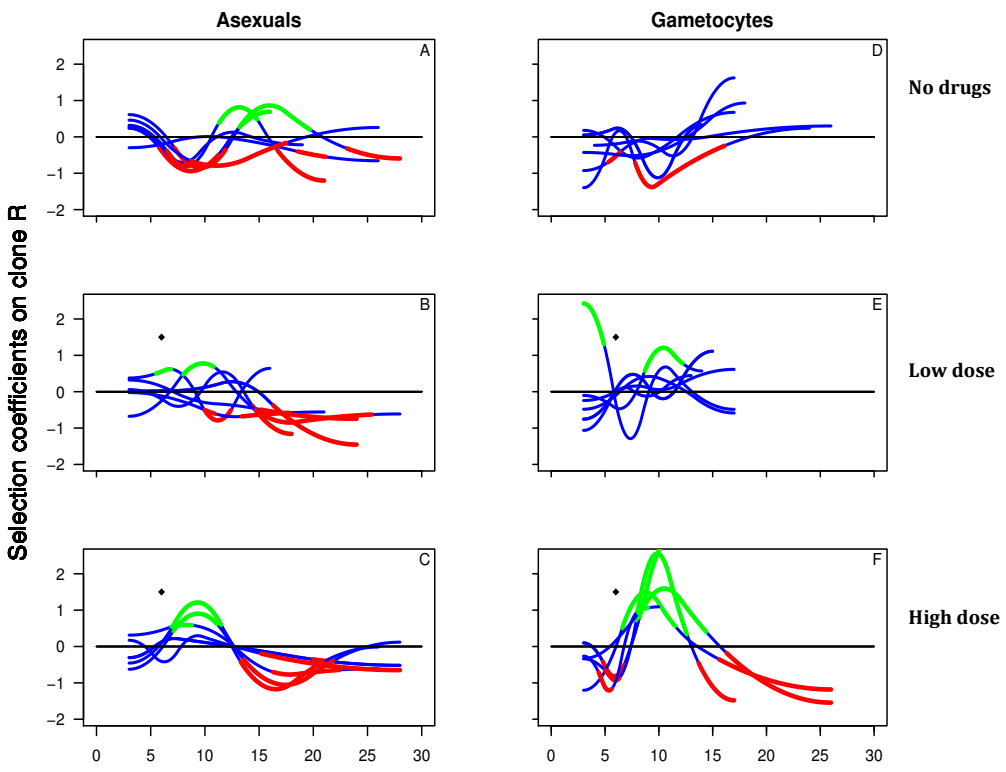

Figure 2.9 Asexual (left panels) and gametocyte (right panels) selection dynamics of clone R for each mouse in mixed infections that were either untreated (upper panels), received low dose treatment (middle panels) or high dose treatment (bottom panels). Lines are the mean selection dynamics with blue segments denoting times when selection is not statistically different from zero, red segments times when selection is statistically less than zero, and green segments times when selection is greater than zero. Selection could be calculated up to the last day that both clones were detectable, which varied between mice.

2.5 Discussion

This study showed that, in the absence of drug treatment, drug-sensitive parasites competitively suppressed resistant parasites, as has been found in previous studies (de Roode et al. 2004; Wargo et al. 2007). Drug treatment led to competitive release of the drug-resistant clone, seen as increased asexual parasite densities. Importantly, competitive release of drug-resistant parasites was more restricted following low dose therapy than high dose treatment, even though the lower dose treatment improved

host health to the same level as high dose treatment. The high dose treatment thus exerted strong positive selection on resistant parasites for little clinical gain. Most importantly, resistant parasites in mixed infections that received high dose drug treatment had the highest transmission potential. Of note is that the drug treatments in this experiment were given on a single day only, while full curative treatment consists of high dose treatment given for four days in a row. Selection under such a treatment regime will be much more extensive than the treatments used in this experiment. Our results confirm the findings in Wargo et al. (2007) and provide proof of principle that less aggressive treatment than is currently recommended could help slow the spread of drug-resistant malaria parasites. Therefore, compared to the treatment regimes currently employed in the field (WHO 2006), milder drug dosages may result in better resistance management without clinical compromise.

However, there need be no simple generality when it comes to drug treatment regimes. Rational treatment design involves taking the within-host ecology into account, which differs greatly between malarious areas. Areas with a low clone multiplicity may require a different approach from highly endemic areas which have high level of multi-genotype infections. In predominantly single-clone infections, competitive release will not play a role in the spread of resistance and low dose drug treatment will thus not have an impact on the spread of resistance, with the exception of selection for *de novo* resistant mutants, which has been demonstrated to be a rare event (Cortese et al. 2002; Wootton et al. 2002; Nair et al. 2003; Hastings 2004; Roper et al. 2004). Similarly, the rationale of reduced drug treatment may apply to a wide range of infectious diseases; however, the ecology of the disease determines whether reduced treatment is a good resistance management tool. In HIV/AIDS infections for instance, *de novo* resistance arises often within the host and transmission is a less important route of acquiring resistance (Wainberg and Friedland 1998). Bacterial infections have an additional complexity that involves lateral gene transfer of resistance genes across bacterial strains and species (Ochman et al. 2000). Therefore, selection for resistance in non-target bacteria within the host may influence the acquisition of resistance in the pathogenic bacteria by means of transfer of resistance genes selected in the non-target bacteria. Thus, while the logic of arguments presented in this study applies to a wide range of diseases, the optimal resistance management

strategy depends on the social context of the disease and may vary for different diseases. Key in all cases is empirical evaluation of treatment regimens.

A new type of analysis for mixed infections of pathogens was introduced here. The selection coefficient analysis makes it possible to directly estimate the strength of selection for resistance imposed by different drug regimes. It also makes it possible to analyse the duration of drug-imposed selection effect. Positive selection for resistance following a high dose treatment lasted for approximately 5 days. Interestingly, the half-life of pyrimethamine in a mouse is estimated to be approximately 4.5 hours (Coleman et al. 1986). If that half-life estimate is correct, our data suggest that positive selection for resistant parasites continued for several days after drug concentrations dropped below 0.001 mg/kg just 2.5 days after drug treatment. Similar effects have been observed in the drug effectiveness of artemisinin derivatives whereby it is suggested that "*the biological effects of the artemisinin compounds extend beyond their presence at therapeutic concentrations in plasma"* (White 1997, pg 1416). What factors drive this positive selection even after drug clearance is unknown. One possibility is that the more abundant resistant clone is capable of temporarily suppressing the less abundant susceptible parasites. Another possibility is that a genotype-specific immune system is inadvertently favouring the resistant parasites by more actively suppressing the previously abundant susceptible parasites (discussed in Wargo et al. 2007). Whatever the explanation, susceptible parasites are not increasing in frequency as soon as drug pressure wears off (Figure 2.1d), which provides an extended fitness advantage to the resistant parasites.

Over the course of infection, both positive and negative selection was exerted on drug-resistant parasites following drug treatment. While resistant parasites experienced positive selection following drug treatment, resistant parasites are selected against after drug effectiveness wears off with the treatment regimes used here. It is interesting that similar selection dynamics as observed in this experiment are seen over the course of a year in *P. falciparum* dynamics in areas with seasonal malaria. In these regions, for instance eastern Sudan, the frequency of resistance genes rises during the rainy season when malaria intensity and hence drug usage is high. When malaria transmission declines, so does the frequency of resistance genes in the parasite population (Abdel-Muhsin et al. 2004; Ord et al. 2007; Babiker 2009).

To our knowledge, this is the first time the kinetics of selection coefficients have been estimated during the course of an infection. The selection coefficients presented here are conservative estimates of the strength of selection. A selection coefficient cannot be calculated on time points when one of the parasite clones is below detection threshold, since selection is a function of the abundance of both clones. However, if parasites of one clone were not detected at an earlier time point, but observed later on in the infection, then these parasites were present earlier on in the infection but below detection threshold. Positive selection on the more abundant clone is very strong on these particular time points, but is not captured in the model. Therefore, the selection coefficients shown in figure 2.5, both positive and negative, may in reality be more extreme.

Estimating the transmission potential of both clones was done based on the gametocyte densities using a density-infectivity function. The exact form of the q-function is not well-established. We used two functions based on different datasets (Carter and Graves 1988; Barnes and White 2005) and our results were qualitatively similar for both q-functions. We also analysed a variety of other hypothetical q-functions (data not shown) and our conclusions were unaffected, unless there was a much higher threshold for infectivity, in which case the resistant clone almost never got transmitted. However, there is a lack of detailed information on the competitive interactions between strains within the mosquito and the impact strain-specific transmission blocking immunity has on strain kinetics. There is evidence that competition between different parasite species within mosquitoes exists (Paul et al. 2002) and so it is likely that there will be competition between presumably ecologically more similar genotypes of the same species. Also, gametocyte infectivity is known to vary through time (Drakeley et al. 2006), not least in response to transmission-blocking immunity. Yet, this analysis is an improvement of the somewhat arbitrary cumulative gametocyte densities through time, and provides a tool to qualitatively compare of the fitness of resistant parasites following different drug treatments.

The susceptible parasite clone in this study showed increased gametocyte densities on the day following high dose drug treatment. On subsequent days, however, gametocyte

densities rapidly dropped. Such effect was not observed following low dose treatment and not clearly seen in the resistant parasite clone. This observation could be the result of so-called drug-induced gametocytogenesis as a response to conditions unfavorable for asexual replication. However, increased gametocyte densities immediately following treatment are not necessarily a result of gametocytogenesis, since gametocyte maturation in *P. chabaudi* is thought to take more than 24 hours (Carter and Graves 1988). An alternative explanation could be reduced gametocyte clearance rates following drug treatment. Increased gametocyte densities following drug treatment have been observed before with pyrimethamine use in *P. chabaudi in vivo* (Buckling et al. 1999a), in *P. falciparum* following chloroquine treatment *in vitro* (Buckling et al. 1999b) and has been suggested multiple times in falciparum malaria in the field following SP drug treatment (Puta and Manyando 1997; Robert et al. 2000; Osorio et al. 2002; Sowunmi and Fateye 2003; Talman et al. 2004; Ali et al. 2006; Sowunmi et al. 2006), although these field data are in the absence of untreated controls difficult to interpret. This facultative response, which interestingly is only seen in response to our high dose drug treatment, could affect the spread of drug resistance since it reduces the relative fitness of drug-resistant parasites; however, it is only observed for a brief period of time and is thus unlikely to have a large overall impact.

Taken together, current treatment regimes may not be the best resistance management strategy. A few recent studies from other disease models come to the same conclusion. In the treatment of both cancer (Gatenby et al. 2009) and *Staphylococcus aureus* infections (Drusano et al. 2009), it appears that reduced treatment eases the proliferation of resistant agents and in the former even reduces mortality. However, at least in the case of malaria, more studies regarding the effects of drug regimes on the competitive interactions between susceptible and resistant parasites and their effect on disease epidemiology are needed, both theoretical and experimental, before such drug regimens can go into clinical trials. Our results are based on rodent malaria parasites. Clearly, mice are not people, therefore these results have to be interpreted with caution. However, in the absence of an alternative to study the within-host ecology of drug-resistant parasites with untreated controls, it is a useful model system to use for proof of concept work as presented here. One study on natural *P. falciparum* infections that does evaluate treated and untreated patients

makes use of data on pregnant women who received intermittent preventative treatment (IPTp) with SP and compared them with a control group of women who elected not to receive IPTp (Harrington et al. 2009). The women who received drug treatment during pregnancy had increased parasitaemia, carried a higher proportion of resistance alleles and presented with lower parasite diversity. While the control group in this study is not truly randomized, it is the best field data on the effect of drug treatment we currently have and shows that drug treatment not only selects for resistance but also increases parasitaemia. The authors attribute this latter effect on competitive facilitation, as described in Wargo et al. (2007). These field data support our results derived from a *P. chabaudi* model that current treatment regimes are not necessarily the best. A rational drug regimen design whereby within-host ecology, a major driver of resistance evolution, is taken into account, may well be the solution to optimal resistance management strategy.

2.6 Appendix

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The key element of the random component in the statistical model is the mean-variance relationship (McCullagh 1983). To estimate this relationship for malaria parasite and gametocyte densities, we conducted additional experimental infections using the resistant clonal parasite lineage on mice that were independent of the experiments. Five replicate samples for asexual parasites in one mouse, and three replicate samples for gametocytes in two different mice were taken every two days during the infection. Due to the amount of blood necessary for conducting DNA and RNA extractions (see material and methods), these were the maximum number of replicates that could be taken from each mouse without influencing parasite kinetics.

The mean-variance relationship for both asexual and gametocyte densities is shown in Figure A1, and suggests a power relationship of the form $\sigma = a\,\mu^b$. This form of a variance to mean relationship indicates that the random component is a type of Tweedie distribution, which belongs to the exponential dispersion model family of distributions. The exact type of distribution is determined by the exponent in the power relationship. For asexuals, the power exponent is b = 1.59, which falls within the range of compound Poisson distributions. For gametocytes, the exponent is b = 2.206, which falls into a different class of distributions based on compound Gamma processes. Given that both values are close to a value of two, we assume that b = 2 for both and refit the a parameter. The assumed value of b=2 fits the data well (dashed line Figure A1), and simplifies the statistical model because it translates into an over-dispersed Gamma distribution. The power of this data is that we now independently estimate the dispersion parameter (a) without having to estimate it from the fitting procedure. The fit dispersion values are a = 0.01415 for asexuals and a = 0.0582 for gametocytes.

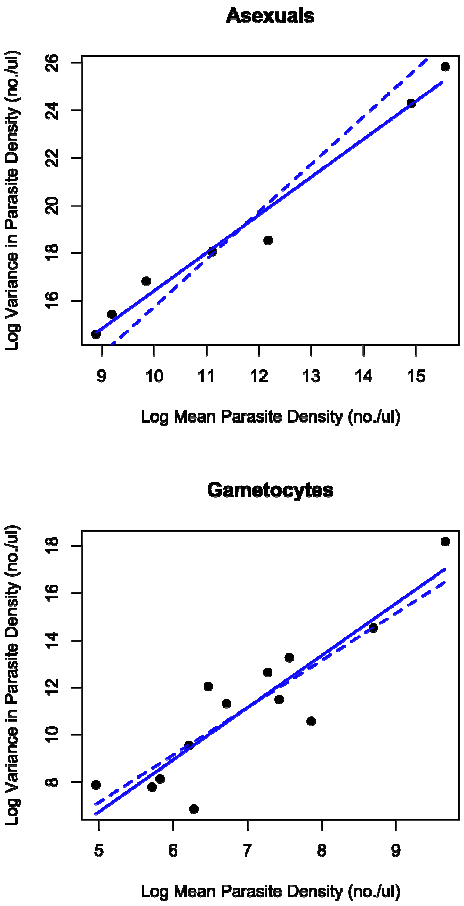

Figure A1 Variance (σ) to mean (μ) relationship for asexual and gametocyte malaria parasites. Points are raw data, solid blue line is the best fit assuming a power relationship $\sigma = a\,\mu^b$, and the dashed blue line is the fit assuming a slope of $b=2$. Parameter estimates for the dispersion parameter are $a = 0.01415$ for asexuals and $a = 0.0582$ for gametocytes assuming a fixed slope of $b=2$.

3. The rarity of drug-resistant parasites in rodent malaria infections affects the kinetics of treatment failure and health outcomes

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3.1 Abstract

Drug resistance is a major problem in malaria control. Previous research has shown that drug treatment provides a great selective advantage to resistant parasites in mixed infections with susceptible parasites. Rare resistant parasites in mixed infections are likely strongly suppressed by more abundant susceptible parasites and for that reason competitive release following drug treatment is expected to be strong. In this study, we infected mice with various ratios of drug-resistant and drug-sensitive *Plasmodium chabaudi* malaria parasites (ranging from 1:1 to $1:10^{-5}$) and were able to track resistant parasites at low frequency through time, at both asexual parasite and gametocyte level. We found that resistant parasites were more suppressed when at low initial density. Drug treatment caused competitive release of the resistant parasites, which was greater for the parasites that started at lower abundance. Competitive release resulted in a second parasite peak, which led to a second wave of anaemia and greater overall infectiousness compared to untreated infections. These results demonstrate that ecological dynamics of mixed malaria infections affect treatment outcome and argue for the importance of highly sensitive detection methods for rare resistant parasite strains in malaria infections.

3.2 Introduction

Drug-resistant malaria parasites are common. For instance, resistance against most widely-used antimalarial drugs has spread globally (Plowe 2009) and the efficacy of the recently widely-employed artemisinin derivatives class drugs (WHO 2008) is declining in South-East Asia (Enserink 2008). Since co-infections are very common in malaria (e.g. Arnot 1998; Babiker et al. 1999; Farnert et al. 2001; Jafari et al. 2004; Zhong et al. 2007), many patients infected with malaria parasites are likely harboring a mixture of parasites of resistant and susceptible to a certain drug. Much work has focused on the molecular and genetic background of resistance (for recent reviews, see Ekland and Fidock 2007; Hayton and Su 2008; Bustamante et al. 2009), but little work has considered the within-host ecological dynamics of resistant parasites in a population of susceptible parasites (Read and Huijben 2009). Yet, these dynamics, and the way in which they are altered by drug treatment are a primary determinant of the rate at which resistant parasites spread through a population (Hastings 2006).

Using a mouse malaria model, previous studies have shown that resistant parasites are competitively suppressed by susceptible parasites, and that following treatment, competitive release occurs (de Roode et al. 2004; Wargo et al. 2007; Huijben et al. *submitted*). This involves an expansion of resistant parasites once the susceptible parasites are removed and can provide great advantage for resistant parasites: not only does the relative fitness of the resistant parasites increase simply due to susceptible parasites being killed by the drug while the resistant ones remain unharmed, it also gives the resistant parasites the opportunity to expand, profiting from the resources that were otherwise used by the susceptible parasites.

In these experiments, the resistant and susceptible parasites were introduced to the host at equal densities. In reality, the frequency of resistance within a host can range from rare (Mookherjee et al. 1999; Bates et al. 2004; Juliano et al. 2007) to near saturation (e.g. McCollum et al. 2007; Mlambo et al. 2007; McCollum et al. 2008; Zhong et al. 2008). Different frequencies within a host can arise from a simultaneous inoculation of different frequencies by the mosquito (coinfection, Nwakanma et al. 2008; Mohanty et al. 2009) or from two sequential infections (super-infection), whereby one parasite strain enters the host when the other strain is already resident (de Roode et al. 2005a). Since the relative fitness of resistant parasites will depend

qualitatively on the extent of competitive release, which might be greater when resistant parasites are rarer, the aim in the work reported here was to study the competitive interactions between drug-resistant and drug-sensitive parasites at varying inoculation abundances to determine whether the extent of competitive release depends on the abundance of resistant parasites.

Furthermore, we were interested in the effect treatment has on the health of hosts that harbor resistant parasites at various abundances with susceptible parasites. When resistant parasites are present in an infection at the time of treatment, they are, by definition, going to cause treatment failure to a certain degree. Treatment failure can result in greater morbidity and even mortality. It can also lead to increased infectiousness, which, in turn, can lead not only to increased malaria transmission, but also to increased transmission of resistant parasites. Therefore, using the *P. chabaudi* mouse model system, we had the following four aims: to establish whether i) competitive suppression, ii) competitive release, iii) the morbidity following drug failure and iv) infectiousness (both overall infectiousness and resistance transmission), is dependent on the abundance of resistant parasites.

3.3 Material and methods

3.3.1 Parasites and hosts

Two genetically distinct clones were used in these experiments, drug-sensitive clone AJ_{5p} (hereafter referred to as clone S) and drug-resistant clone $AS_{6p(pyr\text{-}1A)}$ (hereafter referred to as clone R). Both clones were isolated from thicket rats and subsequently cloned (Beale et al. 1978). Clone R was made resistant by a single high-dose exposure to pyrimethamine (Walliker et al. 1975). Hosts were 15 week old female C57Bl/6 laboratory mice (Charles River Laboratories). This was our first experiment in a new lab, so to test for background variation in mouse health, a group of sham-infected mice were monitored contemporaneously. These mice were eight week old female C57Bl/6 laboratory mice (Charles River Laboratories). All mice were kept on a 12:12 L:D cycle, fed Laboratory Rodent Diet 5001 (LabDiet, PMI Nutrition International) and received 0.05% PABA-supplemented drinking water to enhance parasite growth (Jacobs 1964).

3.3.2 Experimental design, infections and drug treatment

The experiment consisted of either single-clone infections of clone R, or mixed infections of clone R with clone S (Table 3.1). The inoculum of clone R consisted of 10^6, 10^5, 10^3 or 10^1 parasites. In mixed infections, the inoculum of clone S consisted of 10^6 parasites per mouse in all treatment groups, resulting in R:S ratios of 1:1, 1:10, 1:10^3, and 1:10^5, respectively. Control mice were sham-injected with uninfected blood. Half of the mice were drug-treated and the other half left untreated (see below), which totaled to 18 different treatment groups. Each treatment group consisted of five mice, except for the groups with an inoculum of 10^1 resistant parasites which consisted of 10 mice to allow for the possibility that some mice failed to become infected because of stochastic loss due to the low inoculum size (Table 3.1).

Table 3.1 Experimental set-up. Groups were inoculated with clone R in the single-clone infections, or clone R and clone S for the mixed infections. The controls were inoculated with uninfected red blood cells. The inoculum of clone S was always 10^6 parasites, the inoculum of clone R varied from 10^6 to 10^1 parasites. All treatment groups with an inoculum size of 10^1 parasites had 10 mice at the start of the experiment. All other treatment groups consisted of 5 mice. Drug treatment was given on days 6 to 9 post-infection.

	Ratio	Mixed infections			Single-clone infections	
	R:S	R parasites	S parasites	n	R parasites	n
No drugs	1:1	10^6	10^6	5*	10^6	5
	1:10	10^5	10^6	5†	10^5	5
	1:10^3	10^3	10^6	5†	10^3	5
	1:10^5	10^1	10^6	10	10^1	10
	Control	0	0	5		
Drugs	1:1	10^6	10^6	5	10^6	5
	1:10	10^5	10^6	5*	10^5	5
	1:10^3	10^3	10^6	5*†	10^3	5
	1:10^5	10^1	10^6	10**	10^1	10
	Control	0	0	5		

* denotes an excluded mouse, † represents a dead or euthanized mouse

Drug treatment started on day 6 post-infection (PI), which is when pronounced anaemia and weight loss begin to show (Wargo et al. (2007), and see below), and consisted of 8 mg/kg pyrimethamine dissolved in dimethyl sulfoxide (DMSO), administrated by intraperitoneal (i.p.) injection of 50 µl on four successive days. Untreated controls received i.p. injection of DMSO-only contemporaneously.

3.3.3 Monitoring of infections

Weight and red blood cell density of the mice, plus asexual parasite density and gametocyte density of both clones were measured daily (day 3-21 PI) and three times a week thereafter (day 23-49 PI).

For each mouse at any time point, 2 µl of blood was taken by tail snip for red blood cell density measurements using flow-cytometry (Beckman Coulter). Another 5 µl of blood was taken for DNA extraction, which was carried out on the ABI Prism® 6100 Nucleic Acid PrepStation according to manufacturer's instructions. A further 10 µl of blood was taken and lysed immediately for RNA extraction, using the 'RNA Blood-DNA' method on the ABI Prism® 6100 Nucleic Acid PrepStation. Afterwards, RNA was converted to single stranded cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems; Wargo et al. 2006). Both DNA and cDNA were stored at -80°C until quantification. Additionally, a thin blood smear was made of each mouse on each sampling day.

To measure total parasite density (asexual parasites and gametocytes), quantitative PCR was performed on DNA using clone-specific assays. To measure gametocyte density, quantitative PCR was performed on cDNA, using the same clone-specific assays. Asexual parasite density was estimated by subtracting the gametocyte density from the total parasite density. The clone-specific assays have been shown not to amplify parasites from the other clone (Drew and Reece 2007). In brief, the PCR reaction volume of 25 µl for all assays consisted of 7 µl DNA or cDNA, 900 nM forward and reverse clone-specific primers, 250 nM TaqMan® MGB probe (Applied Biosystems) and 1x PerfeCTa™ qPCR FastMix™ (Quanta Biosciences). All reactions were run on the ABI Prism® 7500 Fast System, using the assay: 95°C for 2 minutes, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Quantification was based on serial dilutions of DNA and cDNA standards of known total parasite and gametocyte density, determined beforehand by microscopy (Cheesman et al. 2003).

3.3.4 Statistical analysis

All statistical analyses were performed in R 2.9.0 (R Development Core Team 2009). To summarize the asexual densities through time, which fluctuate over six orders of magnitude with the highest counts occurring on only a few days, we calculated for each mouse the geometric mean asexual parasite density of clone R, or clone S or both for

the entire infection course or for the period following drug treatment (day 10-49). The rate of decline of asexual parasite density of clone S following drug treatment was calculated as the average difference between the log-transformed asexual densities on day 7 and day 10 post-infection. Persistence of each clone was estimated for each mouse as the last day of detectable asexual parasites in the blood. As a measure of transmission potential, the predicted infectiousness was calculated for clone R, clone S and both together for the entire infection course and for the period following drug treatment (day 10-49) as the number of predicted infected mosquitoes out of n=100, using gametocyte densities in the density-infectivity function q1 as described in the general introduction of this thesis. As an estimation of morbidity of acute infection, which was defined as the initial parasite peak and clinical disease episode, the minimum weight and minimum red blood cell densities during this period were used. Furthermore, the minimum red blood cell density and minimum weight during parasite relapse (after day 12 PI) were used as a measurement of the consequences to host health of relapse.

General linear modeling was used with the following factors: *competition* (S present or absent), *drugs* (treated or not) and *R-inoculum* (10^6, 10^5, 10^3, 10^1). Maximal models were fitted first and, beginning with higher order interaction, non-significant terms were sequentially removed to generate minimal models.

Three mice died or were euthanized during the infection (untreated 1:10 R:S, untreated 1:10^3 R:S, drug-treated 1:10^3 R:S) and were removed from further analysis. Another five were also excluded from the analysis. Two of these failed to become infected with resistant parasites from the low inoculum of 10^1 R parasites (both from treated 1:10^5 R:S) and one (drug-treated 1:10^3 R:S) failed to completely respond to drug pressure for unknown reasons. Furthermore, two mice received an inoculum of several orders of magnitude lower than intended, as judged by the kinetics of subsequent growth rates which were therefore not representative of their treatment group (untreated 1:1 R:S, treated 1:10 R:S, Table 3.1).

3.4 Results

In R-only infections, lower doses of clone R resulted in a delay in parasitaemia and gametocytaemia in single infections of clone R (Figure 3.1ab). This delay was about a day for each order of magnitude reduction in dose, as had been observed before in *P. berghei* (Wellde et al. 1966) and *P. chabaudi* (Timms et al. 2001), but had little impact on overall parasite numbers (Table 3.2). Drug treatment had no direct impact on parasite densities of clone R (Figure 3.1cd, Table 3.2), as expected for a resistant clone.

In untreated infections, densities of the resistant clone were suppressed when the sensitive clone was present (Figure 3.2 - left panels, 3.3ab). In all cases, there were lower asexual densities when clone S was present (Figure 3.3a - untreated infections, c*ompetition*: $F_{1,39}$=349, p<0.001), which translated in a reduced predicted infectivity for the resistant parasites (Figure 3.3b – untreated infections, gametocytes, *competition*: $F_{1,39}$=449, p<0.001). Notably, no gametocytes were produced by the resistant clone when it was introduced at the lowest dose in a mixed infection with susceptible parasites (Figure 3.2g, 3.3b).

Close inspection of the left panels of figure 3.2 shows that during the resolution of the initial wave of parasites, densities of clone R dropped in parallel with clone S. Consequently, the fewer R parasites there were in the inoculum, the lower the density achieved before suppression, and hence the greater the competitive suppression (Figure 3.3a – untreated infections, *competition*R-inoculum*: $F_{3,39}$=24, p<0.001).

Table 3.2 Analysis of Variance table of single-clone infections of clone R to test for an effect of drug treatment (*drugs*: treated,untreated), parasite dose (*R-inoculum*: 10^1,10^3,10^5,10^6) and the interaction between them, on geometric mean asexual parasite production and gametocyte production.

	Asexual parasites	Gametocytes
drugs	$F_{1,43}$=0.1, p=0.75	$F_{1,43}$=1.4, p=0.25
R-inoculum	$F_{3,43}$=2.5, p=0.07	$F_{3,43}$=1.3, p=0.29
*drugs*R-inoculum*	n.s.	n.s.

n.s. denotes an insignificant term that was excluded from the model

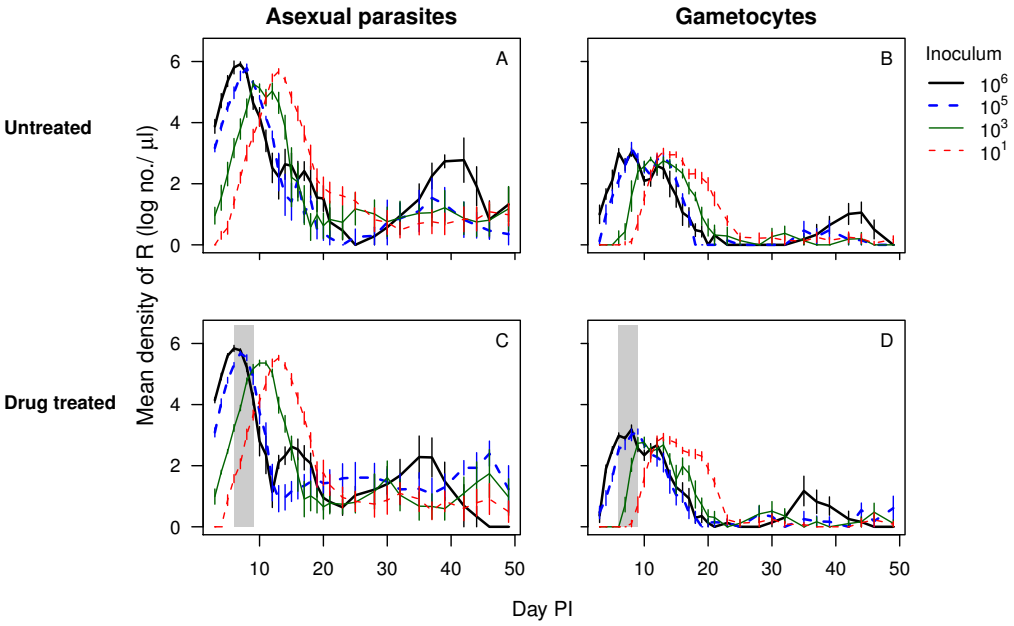

Figure 3.1 Asexual parasite densities (left panels) and gametocyte densities (right panels) in untreated (top panels) and drug-treated (bottom panels) single-clone infections of drug-resistant clone R. The infections were initiated with an inoculum of 10^6 (thick solid black line), 10^5 (thick dashed blue line), 10^3 (thin solid green line) and 10^1 (thin dashed red line) parasites. Drug treatment was given on days 6-9 post-infection as indicated by the shaded area. Data are log-transformed geometric means (± standard error) of 5 mice (inoculum sizes 10^6, 10^5, 10^3) or 10 mice (inoculum size 10^1).

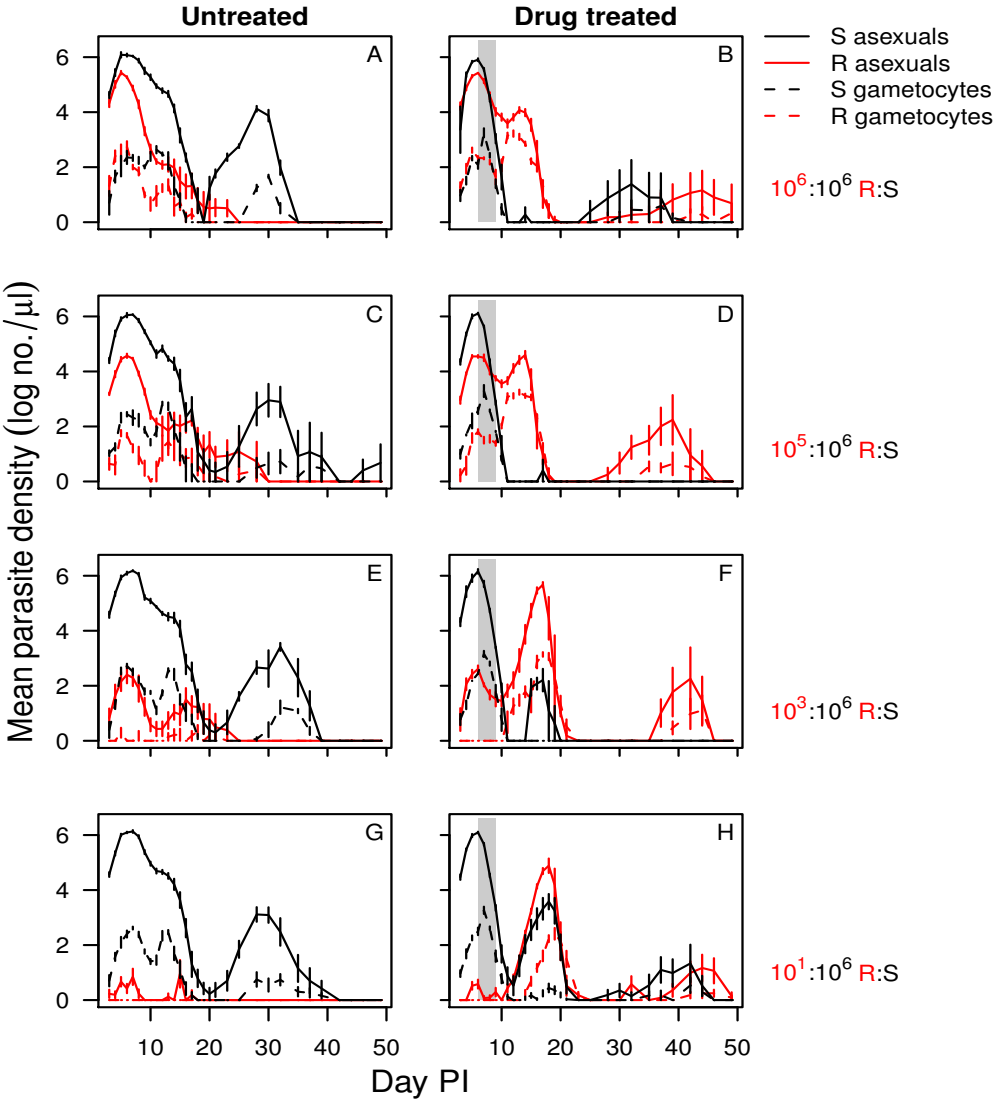

Figure 3.2 Asexual parasite densities (solid lines) and gametocyte densities (dashed lines) of drug-sensitive clone S (red lines) and drug-resistant clone R (black lines) in mixed infections that were untreated (left panels) or drug-treated (right panels). Drug treatment was given on days 6-9 post-infection as indicated by the shaded area. Infections were inoculated with a clone R:S relative abundance of 10^6:10^6 (top row), 10^5:10^6 (second row), 10^3:10^6 (third row) and 10^1:10^6 (bottom row). Data are geometric means (± standard error) of up to 5 mice for R:S abundance of 10^6:10^6, 10^5:10^6 and 10^3:10^6, or up to 10 mice for R:S ratio 10^1:10^6 (Table 3.1).

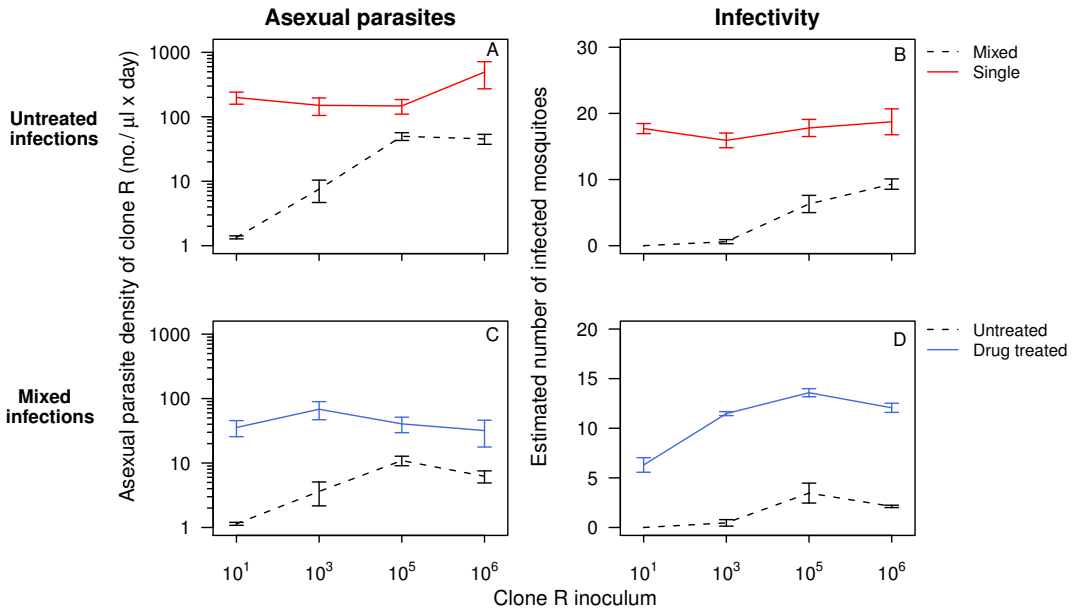

Figure 3.3 Geometric mean asexual parasite density (left panels) and estimated number of infected mosquitoes (from n=100; right panels) of clone R at different initial parasite dosages (x-axis) in untreated infections (upper panels) of single-clone (red solid line) and mixed-clone (black dashed line) infections, and in mixed-clone infections (bottom panels) with drug-treated (blue solid line) and untreated (black dashed line) infections post-treatment (day 10-49). Data are means of up to 5 mice (R inoculum sizes 10^6, 10^5, 10^3) or up to 10 mice (inoculum size 10^1; table 3.1). Asexual parasite densities are plotted in a log-scale, note the y-axis varies between the right panel plots.

3.4.1 Post-treatment kinetics

As expected, the density of resistant parasites at the start of treatment was determined by the number inoculated, so that the initial inocula of 10^6, 10^5, 10^3, 10^1 had, by the time of treatment (day 6 PI), become $10^{5.4}$ /µl, $10^{4.6}$/µl, $10^{2.6}$/µl, $10^{0.7}$/µl respectively.

In mixed infections, susceptible parasites were rapidly cleared by drugs (Figure 3.2 – right panels). As a result, the initially suppressed populations of resistant parasites were able to expand after drug treatment (Figure 3.3cd – mixed infections asexual parasites, *drugs*: $F_{1,36}$=107, p<0.001), which caused a pronounced second peak of parasitaemia (Figure 3.2 – right panels). The extent of this competitive release was greater when resistant parasites had started out at lower abundances (Figure 3.3cd - mixed infections, asexual parasites, *drugs*R-inoculum*: $F_{3,36}$=4.1, p=0.013). Thus, resistant parasites had the biggest disadvantage when present at low abundance in

untreated infections, and so gained proportionately the most when susceptible parasites were removed by drug treatment.

Competitive release translated into large increases in transmission potential, with drug treatment greatly increasing the predicted infectivity of the resistant parasites (Figure 3.3d – mixed infections, R-infectivity, *drugs*: $F_{1,36}$=366, p<0.001; *drugs***R-inoculum*: $F_{3.36}$=6.9, p=<0.001).

Competitive suppression in untreated infections eliminated the resistant parasites on average by day 16 (range: day 3-28; Figure 3.2 – left panels), whereas clone R persisted for over 2.5 times as long, on average up to day 42, in the absence of competition (range: day 18-49; Figure 3.1 – untreated infections, *competition*: $F_{1,42}$=124, p<0.001). Drug treatment removed the force of suppression, which allowed the resistant parasites to persist for twice as long as in the absence of treatment (mean: 32 days, range: day 15-49; Figure 3.2, right panels –, *drugs*: $F_{1,39}$=27, p<0.001). The initial abundance of clone R did not affect its ability to persist in mixed infections (*R-inoculum*: $F_{3,39}$=1, p=0.40) .

Unexpectedly, the impact of drugs on the kinetics of the susceptible clone was affected by the number of resistant parasites at the start of the infection. Even though the rate of reduction due to drug treatment was the same for all drug-treated groups ($F_{3,18}$=0.4, p=0.78), the release of resistant parasites resulted in a recrudescence of susceptible parasites in the lower abundance groups (Figure 3.2 – right panels). This second peak of susceptible parasites resulted in a higher production of asexual S parasites after treatment in infections with a low number of R parasites at the outset (drug-treated mixed infections, *R-inoculum*: $F_{3,18}$=18, p<0.001). However, these recrudesences did not translate into a higher predicted infectivity of clone S (Figure 3.2fg, $F_{3,18}$=0.9, p=0.45).

3.4.2 Overall parasite burden and transmission potential of mixed infections

The total number of parasites present throughout the entire infections (R+S parasites, days 3-49) were reduced by drug administration (Figure 3.4a – mixed infections, *drugs*: $F_{1,36}$=42, p<0.001), despite the increase in resistant parasites (Figure 3.4c). However, drug treatment least effectively reduced total parasite burden when

resistant parasites were rare at the time of treatment (*drugs*R-inoculum*: $F_{3,36}$=3.5, p=0.02), due to a greater abundance of S parasites that occurred during the post-treatment release (Figure 3.2h).

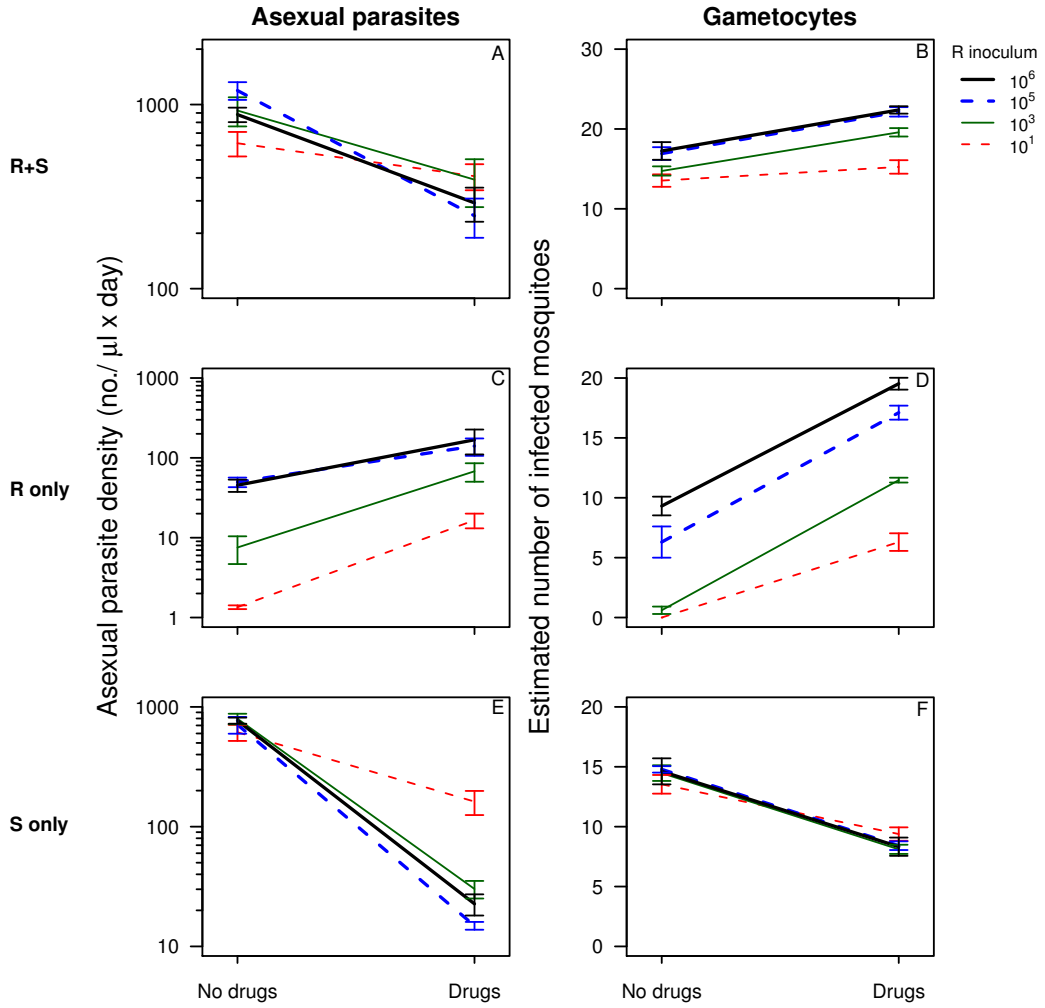

Figure 3.4 Geometric mean asexual parasite density (left panels) and estimated number of infected mosquitoes (from n=100; right panels) of clone R with clone S combined (top panels), clone R only (middle panels) or clone S only (bottom panels) in untreated and drug-treated mixed infections that were initiated with a clone R inoculum of 10^6 (thick black solid line), 10^5 (thick blue dashed line), 10^3 (thin green solid line) and 10^1 (thin red dashed line) parasites. The inoculum of clone S was 10^6 in all cases. Data are means of up to 5 mice (R inoculum sizes 10^6, 10^5, 10^3) or up to 10 mice (R inoculum size 10^1; Table 3.1). Asexual parasite densities are plotted in a log-scale, note the y-axis varies between plots.

Moreover, drug treatment increased total gametocyte numbers, which translated into a higher overall infectivity (Figure 3.4b – mixed infections, *drugs*: $F_{1,39}$=32, p<0.001).

The magnitude of this increase was not strongly related to the rarity of resistant parasites (*drugs*R-inoculum*: $F_{1,36}$=2.5, p=0.077), although the more abundant clone R was in the initial inoculum, the higher the overall infectivity (*R-inoculum*: $F_{3,39}$=19, p<0.001). The increase in total infectivity in drug-treated infections was caused by a strong increase in predicted transmission for the resistant parasites (Figure 3.4d), opposed to a smaller decrease in predicted transmission for the susceptible parasites (Figure 3.4f).

The total transmission potential for the resistant parasites over the entire infection was dependent on both drug treatment and abundance in a mixed infection. Drug treatment greatly increased the transmission potential of the resistant parasites (mixed infections, *drugs*: $F_{1,36}$=270, p<0.001). While the relative increase in transmission potential as a result of drug treatment was greater for low abundance parasites than for high abundance parasites (*drugs*R-inoculum*: $F_{3,36}$=51, p<0.001), more abundant R parasites at the outset had a greater transmission potential overall (*R-inoculum*: $F_{3,36}$=51, p<0.001). In fact, the transmission potential for these more abundant R parasites was as large as it was in the absence of competition. For the R parasites that started out at lower abundances however, the overall transmission potential was not as high as it would have been in the absence of competition (drug-treated infections, *competition*R-inoculum*: $F_{3,37}$=5.5, p=0.003).

3.4.3 Host health

P. chabaudi infections caused significant weight loss and anaemia when comparing them to sham-injected controls (Figure 3.5ab). The acute infection was more severe in mixed infections of clone R and S than for single-clone infections with clone R only (*competition*: minimum weight, $F_{1,79}$=78, p<0.001; minimum RBC density, $F_{1,79}$=39, p<0.001), likely driven by the greater virulence of clone S (de Roode et al. 2004; Bell et al. 2006; Wargo et al. 2007; Huijben et al. *submitted*). Drug treatment, as expected, significantly reduced the severity of the acute infection (mixed infections, *drugs*: minimum weight, $F_{1,39}$=23, p<0.001; minimum RBC density, $F_{1,39}$=73, p<0.001). The abundance of clone R did not have an impact on the virulence of acute infection.

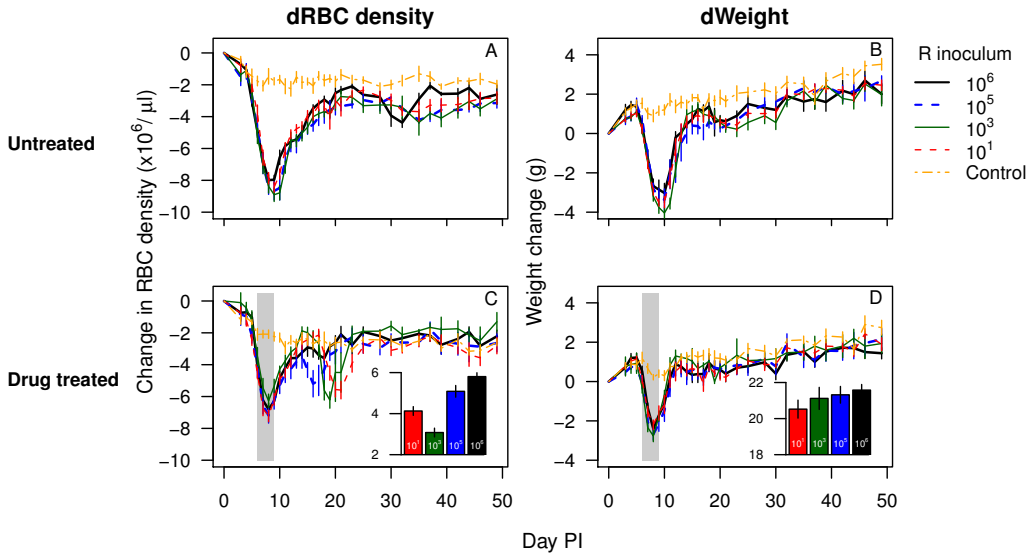

Figure 3.5 Change in red blood cell densities (left panels) and weight change (right panels) from initial (day -1) values of mixed infections that were left untreated (top panels) or drug-treated (bottom panels). Drug treatment was given on days 6-9 post-infection as indicated by the shaded area. The infections were initiated with an R inoculum of 10^6 (thick solid black line), 10^5 (thick dashed blue line), 10^3 (thin solid green line) and 10^1 (thin dashed red line) parasites. The inoculum of clone S was 10^6 in all cases. Sham-injected mice are shown in thin dotdashed orange line. The inset graphs show the minimum red blood cell density (x10^6/µl) (c) and minimum weight (gram) (d) reached after drug treatment. Data are means (± standard error) of up to 5 mice (R inoculum sizes 10^6, 10^5, 10^3) or up to 10 mice (inoculum size 10^1; Table 3.1).

However, the drug treatment induced a second wave of anaemia caused by the competitive release of resistant parasites (see above and Figure 3.5c). No relapses were observed in any of the single clone R infections (data not shown). Drug treatment caused more severe anaemia during this period of post treatment relapse than in untreated infections, but only in the infections where resistant parasites were at low abundance (mixed infections, minimum RBC density during relapse, *drugs***R-inoculum*: $F_{3,36}$=8.0, p<0.001). The parasite relapse did not significantly affect weight during this period (Figure 3.5d). In fact, for all abundances, drug-treated infections had higher weights than untreated infections during the post treatment relapses (mixed infections, *drugs*: $F_{1,39}$=9, p=0.005). The abundance of clone R did not affect the minimum weight during relapse (*R-inoculum*: $F_{3,39}$=0.7, p=0.55).

3.5 Discussion

We found that the fitness of resistant parasites was density-dependent: the more abundant the competitor at the start of an infection, the more suppressed the resistant clone was. The parasite population appeared to be regulated non-specifically during the acute infection; when the more abundant susceptible parasites declined in density, the less abundant resistant parasites declined as well. These dynamics are most likely regulated by resource abundance (such as red blood cell density, see Mideo et al. 2008), although innate, non-specific immunity may also play a role (reviewed in Mideo 2009). Drug treatment released the competitive suppression of the resistant parasites with greater expansion following treatment of the infections where resistance was rare. Drug treatment of mixed infections did not necessarily benefit the host; even though drug treatment alleviated acute phase disease, drug-induced release of the minority resistant parasites resulted in a second wave of parasites that caused another bout of anaemia. Moreover, the treated mixed infections had a slightly higher transmission potential than untreated infections. In conclusion, drug treatment of mixed infections gave rise to an increased overall transmission and increased relative fitness of resistant parasites, without being much more beneficial to host health.

The within-host dynamics following an inoculum of 10^1 resistant parasites mixed with 10^6 susceptible parasites may resemble the dynamics following a *de novo* resistance mutational event. There is very little understanding of the dynamics between wild-type and resistant mutants following a mutational event within the host. The actual occurrence of *de novo* mutations is rare, only a handful of times for both chloroquine and sulphadoxine-pyrimethamine resistance (reviewed in Plowe 2009). Reasons for this rare occurrence of *de novo* mutations in the field are unknown, but are thought to be due to multiple mutations necessary for a viable mutant. Competition will also play an important role in determining whether a *de novo* resistant mutant can rise to transmissible densities. This study showed that resistant parasites that started out at a 10^1 inoculum did not produce a single detectable gametocyte in the absence of treatment. Many infections in Africa are left untreated, as opposed to Southeast Asia, where most infections, due to low transmission intensity, are symptomatic and thus more often treated (Pongtavornpinyo et al. 2008). This could (partly) explain why resistance seems to originate in Southeast Asia rather than Africa for all widely employed drugs. In this study, the rarest resistant parasite started in the inoculum at a

frequency of 10^{-5} and was at similar frequency at the time of treatment. Since the mutation rate for *P. falciparum* is though to be around 10^{-9} (Paget-McNicol and Saul 2001), the frequency of resistant mutants in an infection is likely much lower than 10^{-5}. Further experiments with resistant parasites at lower frequencies, for instance by 'seeding' the resistant parasites later in the infection at high abundance of susceptible parasites, could reveal the intensity of suppression at these extremely rare abundances.

Due to the design of the study, we are unable to distinguish between parasite density- and frequency-dependence, as frequency changed with density. However, our conclusions likely apply to both. A previous study by de Roode et al. (2005a) showed increased suppression of clone R when it was introduced in the host at the same initial density three days after clone S. Although absolute densities were different, at time of inoculation there were approximately three orders of magnitude difference between clone R and S; a frequency similar to the 10^3:10^6 inoculum in this study. The subsequent dynamics were very similar in both experiments. These results confirm our expectations, though further work should indicate whether the results found in this study are indeed frequency dependent.

Drug treatment increased virulence (measured as increased anaemia) in infections where resistant parasites were present at low initial abundance, but was actually beneficial in infections that started with a higher abundance of resistant parasites. This result suggests that the frequency of resistant parasites in a mixed infection is an important determinant of clinical outcome and in non-intuitive ways. In particular, it may be dangerous for the patient to aggressively treat an infection containing rare, possibly undetectable resistant parasites. An example of this is an increased parasitaemia in pregnant women taking intermittent preventative treatment (IPTp) of SP in an area with widespread SP resistance compared to women opting out of IPTp (Harrington et al. 2009). This raises the question of tailoring treatment regimes to the presence or absence of resistant parasites in the population. High sensitivity methods such as ultradeep pyrosequencing or heteroduplex tracking assays (Kwiek et al. 2007; Bushman et al. 2008) in field trials could provide very useful information on the presence and dynamics of resistant parasites after drug treatment in field settings. Moreover, such highly sensitive diagnostics could be useful in the western world,

where such techniques are more readily available and affordable, for examining infected travelers returning from malaria endemic areas. Detecting resistant parasites that are low in abundance could avoid severe relapses in these non-immune patients.

An increased transmission potential was found for drug-treated mixed infections compared to equivalent but untreated infections. This increase was due to the release of resistant gametocytes. If this occurs in *P. falciparum* infections, it has important consequences for malaria control: not only would transmission of resistant parasites increase as a result of drug failure, but so would overall malaria transmission. These argue for a better understanding of the genetic make-up of malaria infections. The most dramatic increase in overall gametocyte production was observed in the infections that started out with the higher abundance of resistant parasites. Since these abundances are above the detection threshold of standard PCR methods that are already in use in the tropics today, we argue for the implementation of routine screening for resistant parasites in malaria patients as soon as the molecular background is known. Such routine screening might prove to be especially valuable in areas where malaria eradication is the goal.

Unexpectedly, the drugs worked less efficiently on the susceptible parasites when resistant parasites were present at low frequencies. This result means there is an even stronger argument for identifying the genetic build-up of the infection prior to drug treatment: if resistant parasites are present at low frequencies, not only will resistant parasites greatly increase in density in the infection, but so will susceptible parasites. We do not understand what caused the relapse of the susceptible clone. The drug treatment given was evidently insufficient to fully clear the population of susceptible parasites. However, infections with the susceptible clone alone never have such a relapse (e.g. Wargo et al. 2007), which therefore strongly suggests that something to do with the expanding population of resistant parasites allows the sensitive parasites to relapse as well. A possible explanation is that the expanding population secretes immune suppressants which facilitate the growth of the susceptible clone. Nevertheless, the relapse of susceptible parasites did not translate in a much increased gametocyte density; therefore, the relative fitness of the resistant parasites was mostly unaffected by this relapse of clone S.

The occurrence of a second peak of parasites after treatment, as seen in these experimental infections, is frequently observed in the field, often referred to as 'recurrence'. These recurrences could be the result of recrudescence from the existing infection (i.e. treatment failure), or of re-infections, which especially happens in high transmission areas. Therefore, standard practices for antimalarial trials is the use of a so-called 'PCR correction' (Snounou and Beck 1998), whereby the infection is genotyped before and after treatment to conclude whether recurrence is due to recrudescence or reinfection. The accuracy of genotyping methods has been debated: much of the outcome depends on arbitrary decisions that researchers have to make regarding the number of markers used, the statistical analysis, and subjective criteria on the exact allele frequencies at which to differentiate between new and recrudescing infections (Greenhouse et al. 2007; Ashley et al. 2008; Liu et al. 2008). Also, the visual inspections of band sizes appears to be subjective and variable (Rouse et al. 2008) and will not differentiate between small amplicon length differences or sequences (Juliano et al. 2009). Most importantly, the sensitivity of these methods to pick up minority strains is highly doubted (Liu et al. 2008; Juliano et al. 2009). Alleles present in less than 20% of the population cannot be reliably demonstrated (Juliano et al. 2009), yet, the present study shows a profound recurrence from a minority strain lower than 0.001% at the time of treatment. PCR correction could thus seriously underestimate treatment failure. The use of new methods such as heteroduplex tracking assays and ultradeep pyrosequencing could be of great value in significantly increasing our understanding of recurrent parasites (Kwiek et al. 2007; Bushman et al. 2008).

The ultimate objective of many malaria policy makers and funding bodies dealing with malaria control nowadays is malaria elimination or even eradication. The proposed programs to achieve this goal are to a large extent dependent on drug treatment (Roll Back Malaria 2008; Feachem and Malaria Elimination Group 2009), yet we know surprisingly little about the effect such an extensive drug administration has on the spread of resistance. The data that exists on mass drug administration provides sometimes conflicting results, though mass drug treatments are generally assumed to promote the spread of drug resistance (von Seidlein and Greenwood 2003). We also know resistance can spread very fast once it has arisen (Plowe 2009). It is therefore important that we better understand the dynamics of resistant parasites. The data in this study clearly show that within host dynamics prior to drug treatment can

influence treatment outcome, both from a patient-perspective and for the transmission of resistance. Highly sensitive techniques assessing parasite population dynamics could provide very useful information in clinical trials, giving (i) a better insight in competitive interactions between resistant and sensitive *P. falciparum* strains, (ii) a reduction of the spread of drug resistance and overall transmission and (iii) an improvement of treatment regimes on a patient-specific level. Failure to incorporate resistant parasite dynamics in eradication programs might not only result in a failure of malaria eradication, but could also make the health-situation worse than it was prior to intervention.

4. Competitive release of resistant parasites following removal by chemotherapy of susceptible parasites from the same clonal lineage

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4.1 Abstract

One of the aims of radical parasitological treatment is to reduce the probability of resistant malaria mutants arising *de novo* within a patient. However, such treatment regimes will maximally up-select any resistant mutants that are present. Previous research has shown that resistant parasites present at a low frequency in infections with unrelated sensitive parasites were greatly advantaged by competitive release following drug treatment. *De novo* resistant mutants would also be at very low frequency in a mixed infection with their sensitive progenitors. Using the *Plasmodium chabaudi* malaria model, we test the hypothesis that resistant parasites at low frequency in infections of sensitive parasites, both originating from the same ancestral lineage, also result in a pronounced parasite relapse following drug treatment. We found that drug treatment induced distinct parasite relapses in both genetically related and genetically unrelated mixed infections of drug-resistant and drug-sensitive parasites. This finding suggests that competitive release following drug treatment may play an important role in up selecting *de novo* resistant mutants once they have arisen in the infection.

4.2 Introduction

Current antimalarial treatment guidelines advise radical parasitological treatment (aimed at rapid elimination of all parasites) to reduce the probability that *de novo* resistant mutations occur in infections (WHO 2006). Yet, much experimental evidence shows that resistance in the laboratory is most readily obtained by exposing large numbers of malaria parasites to high drug dosages (discussed in Peters 1987). Such a selection treatment regime used to select for resistant parasites in the lab is similar to radical parasitological treatment in malaria patients. Drug treatment which aims at radical parasitological cure, using high drug dosages over a longer time period, decreases the likelihood of parasites with the relevant mutations to occur by means of reducing absolute parasite numbers. However, if a resistant mutant has arisen in the infection, such radical treatment is expected to provide the greatest possible selection for these resistant mutants since it will eliminate competition by the susceptible progenitor parasites. Therefore, in terms of resistance management, radical parasitological treatment is likely a two-edged sword. How these two contrasting forces play out has yet to be determined.

Drug-resistant parasites are likely suppressed when in competition with susceptible parasites due to a cost of resistance (Walliker et al. 2005; Felger and Beck 2008; Babiker et al. 2009). When drug treatment is given to a mixed infection of drug-resistant and drug-sensitive parasites, the relative fitness of resistant parasites increases because of a survival advantage resistant parasites have over susceptible ones. Additionally, the resistant parasites can be released by expanding into the ecological space that was previously occupied by the susceptible parasites. This competitive release could further increase the relative fitness of resistant parasites (Hastings 2003; de Roode et al. 2004; Wargo et al. 2007; Huijben et al. *submitted*). The extent of competitive release may be dependent on the strength of competitive suppression, which is expected to be much greater between *de novo* resistant mutants and its sensitive progenitor since they will likely occupy identical niches. Such competitive suppression may be particularly strong against *de novo* resistant mutants since little evolutionary time has passed to accumulate compensatory mutations (Walliker et al. 2005).

Understanding the initial selection process for resistant mutants is of critical importance for designing treatment regimes that reduce the likelihood of drug resistance arising. A previous experiment with resistant parasites at low frequency in the infection has shown that pronounced competitive release occurs following drug treatment (Chapter 3). However, the resistant parasites in that experiment were genetically distinct from the susceptible parasites and therefore differ at many loci. This means they can potentially occupy different niches and be differentiated by the immune system. Experiments whether competitive release occurs when drug-resistant and susceptible parasites are from the same clonal lineage have yet to be conducted. Here we report such a test.

Pyrimethamine-resistant and susceptible *Plasmodium chabaudi* parasites of the same clonal lineage differ in only a single nucleotide (Cheng and Saul 1994). So far as we are aware, standard PCR techniques are not capable of accurately quantifying single nucleotide polymorphisms (SNPs) at a frequency lower than 5% (Hunt et al. 2005; Cheesman et al. 2007). A mixed infection of a pyrimethamine-resistant *P. chabaudi* clone with its susceptible ancestral lineage can thus not be quantified for each clone separately. However, the experiment described in chapter 3 showed a pronounced relapse of resistant parasites following drug treatment of an infection with low-frequency resistant parasites competing with genetically distinct susceptible parasites. Therefore, to test our hypothesis that mixed infections of rare resistant parasites in competition with susceptible parasites from the same genetic background also have significant relapses of resistant parasites following drug treatment, we studied the total parasite kinetics following drug treatment. If competitive release of resistant parasites occurs following treatment, we expect to observe a pronounced relapse peak. Such distinct parasite relapses were indeed found in both genetically related and genetically unrelated mixed infections of drug-resistant and drug-sensitive parasites. This suggests that competitive release following drug treatment may play an important role in up selecting *de novo* resistant mutants once they have arisen in the infection.

4.3 Material and Methods

4.3.1 Parasites and hosts

Parasites from three clonal lineages were used in these experiments, drug-sensitive clone AJ_{8p} (hereafter referred to as clone AJ_{sens}[1]), drug-resistant clone $AS_{8p(pyr\text{-}1A)}$ (hereafter referred to as clone AS_{res}^{*}) and drug-sensitive ancestral lineage of clone AS_{res}, $AS_{13p(sens)}$ (hereafter referred to as clone AS_{sens}). Clones AJ_{sens} and AS_{sens} were originally isolated from thicket rats and subsequently cloned (Beale et al. 1978). Clone AS_{res} was derived from an ancestor of AS_{sens} (AS_{35}) and made resistant by exposing it to a high dose of pyrimethamine in a single passage (Walliker et al. 1975). Since the splitting of AS_{res} from AS_{sens}, both clones have been passaged 25 times through vertebrate hosts (AS_{res} through 16 mice and 9 thicket rats, AS_{sens} through 24 mice and 1 thicket rat). Clone AS_{res} has been passaged 5 times through a mosquito vector, clone AS_{sens} 3 times. Clone AS_{res} has been exposed to pyrimethamine in two additional passages following the selection process.

Hosts were 10 week old female C57Bl/6 laboratory mice (Charles River Laboratories). All mice were kept on a 12:12 L:D cycle, fed Laboratory Rodent Diet 5001 (LabDiet, PMI Nutrition International) and received 0.05% PABA-supplemented drinking water to enhance parasite growth (Jacobs 1964). used

4.3.2 Experimental design, infections and drug treatment

Mixed infections of AS_{res} + AJ_{sens} (treatment groups 1 and 5), and AS_{res} + AS_{sens} (treatment groups 2 and 6) were initiated with an inoculum consisting of 10^6 parasites of the susceptible clone (AS_{sens} or AJ_{sens}) and approximately 10 parasites of clone AS_{res} (Table 4.1). Treatment groups with single-clone infections of clone AS_{res} with an inoculum of ~10 parasites were included to establish infection success following the seeding event in the absence of competition (treatment groups 3 and 7), and single-clone infections of clone AS_{sens}, with an inoculum of 10^6 parasites, to determine the rate

[1] Note that clone AJ_{sens} is of the same clonal lineage as clone S in other chapters in this thesis and clone AS_{res} is of the same clonal lineage as clone R elsewhere in this thesis. For clarity, clone annotations other than those used elsewhere in the thesis had to be applied in this chapter since the otherwise nonsensical concept of a susceptible clone R had to be used.

at which susceptible parasites could relapse, for instance through the generation of *de novo* mutations (treatment groups 4 and 8). Half of the mice received drug treatment (treatment groups 5-8), which was given on days 6-9 post-infection (PI) with 8 mg/kg pyrimethamine dissolved in 0.05 ml DMSO. The other half was left untreated and received 0.05 ml DMSO only (treatment groups 1-4). Both parasites and drugs were administered by an intra-peritoneal injection. The treatment groups that received clone AS_{res}, either in a mixture or as a single-clone infection, consisted of 9 mice to allow for the possibility that some mice would fail to become infected with clone AS_{res} due to stochastic loss due as a result of the low inoculum size. Treatment groups with a single-clone infection of clone AS_{sens} consisted of 5 mice (Table 4.1).

Table 4.1 Experimental setup. Infections consisted of either single-clone infections of clone AS_{res} and clone AS_{sens}, or mixed infections of AS_{res} + AJ_{sens} and mixed infections of AS_{res} + AS_{sens}. Inoculations with clone AS_{res} consisted of 10^1 parasites, inoculations of clones AS_{sens} and AJ_{sens} of 10^6 parasites. All treatment groups containing AS_{res} parasites consisted of nine mice, the treatment groups containing single-clone AS_{sens} consisted of five mice.

Treatment group	Sensitive clone (parasite dose: 10^6)	Resistant clone (parasite dose: 10)	n
	No drugs		
1	AJ_{sens}	AS_{res}	$9^{\dagger}$
2	AS_{sens}	AS_{res}	$9^{\dagger\dagger}$
3	-	AS_{res}	9*
4	AS_{sens}	-	5
	Drug-treated		
5	AJ_{sens}	AS_{res}	$9^{\dagger\dagger}$*
6	AS_{sens}	AS_{res}	9
7	-	AS_{res}	9
8	AS_{sens}	-	5

† denotes a dead or euthanized mouse, * indicates an uninfected mouse

4.3.3 Monitoring of infections

Body mass and red blood cell density of the mice, plus total parasite density were measured daily from days 3 to 26 PI and subsequently on days 28, 31 and 33. For each mouse, body mass was measured (to the nearest 0.1 gram), 2 µl of blood was taken by tail snip for red blood cell density measurements using flow-cytometry (Beckman Coulter) and a thin blood smear was made for estimating parasitaemia. Parasite density was calculated by multiplying parasitaemia with red blood cell density. The change in body mass in percentage through time was calculated for each mouse from a

baseline value, for which the mean body mass on the day prior to infection and day 3 post-infection was used.

On days 5-8 and 17-21 PI, another 5 µl of blood was taken from all AS_{res} + AJ_{sens} infections (treatment groups 1 and 5) for DNA extraction, which was carried out on the ABI Prism® 6100 Nucleic Acid PrepStation according to manufacturer's instructions. DNA was stored at -80°C until quantification. DNA was extracted from these mice to confirm mixed infections during acute phase and establish the presence of parasite clones during the relapse phase. Since clones AS_{res} and AS_{sens} in mixed infection could not be distinguished by qPCR, this procedure was not done on mice with AS_{res}+AS_{sens} infections. From these mice and the single clone infections, 5 µl of blood was also taken but immediately discarded. An AS-specific and AJ-specific qPCR assay was performed on the DNA samples, as described elsewhere (Chapters 2,3, 5 and 6), using the primer and probe sequences of Drew and Reece (Drew and Reece 2007).

4.3.4 Statistical analysis

To summarize the parasite densities through time, which fluctuate over six orders of magnitude with the highest counts occurring on only a few days, the geometric mean parasite density post-treatment (day 7-33 PI) and during parasite relapse were calculated. A relapse was defined as a second peak in parasite densities following a period of the parasites being below detection threshold. Additionally, peak parasite density and time of the peak of relapse was taken.

As an estimate of virulence of the acute infection (defined as the initial parasite peak and clinical disease episode), the maximum loss in body mass and minimum red blood cell densities during this period were obtained. Furthermore, the minimum red blood cell density and minimum weight during parasite relapse were used as a measurement of virulence of relapse.

General linear modeling was used to fit the following factors: *clone* (AS_{res}, AS_{sens} or AJ_{sens}) and *drugs* (treated or untreated). The data were analyzed separately for single and mixed infections. Maximal models were fitted first and non-significant interaction terms were removed to generate minimal models. All statistical analyses were performed in R 2.9.0 (R Development Core Team 2009).

Five mice died or were euthanized during the infection: two in the drug-treated AS_{res} + AJ_{sens} mixed infection group, one in the untreated AS_{res} + AJ_{sens} mixed infection group and two in the untreated AS_{res} +AS_{sens} mixed infection group. Additionally, one mouse in the drug-treated AS_{res} + AJ_{sens} mixed infection group failed to become infected, as well as one mouse in the single AS_{res} infections group. These mice were excluded from the analysis (Table 4.1).

4.4 Results

In 17 out of the 18 mice that were inoculated with ~10 AS_{res} parasites, infection was successfully established. AS_{res} was also detected by qPCR for at least one day in all 14 co-infections with clone AS_{res} + AJ_{sens}. Thus, despite the low parasite dose, the inoculum of AS_{res} was successful at establishing an infection.

As expected from a previous experiment (Chapter 3), a parasite dose of ~10 AS_{res} parasites resulted in a later peak parasitaemia compared to the inoculation of 10^6 AS_{sens} parasites (Figure 4.1ab). As expected, clone AS_{res} was resistant to pyrimethamine: parasite density between drug-treated infections was similar to untreated infections (Figure 4.1a). Clone AS_{sens} was susceptible to drugs, showing much lower mean parasite density in drug-treated infections compared to untreated infections (Figure 4.1b, *drugs* x *clone* interaction: $F_{1,23}$=122, p<0.001). Consequently, drug treatment did not have an impact on maximum red blood cell loss in AS_{res} infections, whereas it did in AS_{sens} infections (Figure 4.1cd, *drugs* x *clone* interaction: $F_{1,23}$=9.1, p=0.006). Drug treatment slightly reduced loss in body mass in AS_{sens} - infections, while it did not for AS_{res} infections, though no significant interaction was observed (Figure 4.1ef, *drugs* x *clone* interaction: $F_{1,23}$=0.8, p=0.38)

4.4.1 Relapses

Drug-treated AS_{res} + AJ_{sens} infections showed a pronounced relapse (Figure 4.2a), as seen in previous experiments (Chapters 2, 3 and 6). This relapse was observed in all six infections. PCR analysis of the relapses demonstrated that the large majority of the parasites in the relapse were AS_{res} parasites. As observed in previous experiments,

clone AJ_{sens} was also detected in the relapses in 5 out of 6 infections; though at parasite densities that were two orders of magnitude lower (data not shown).

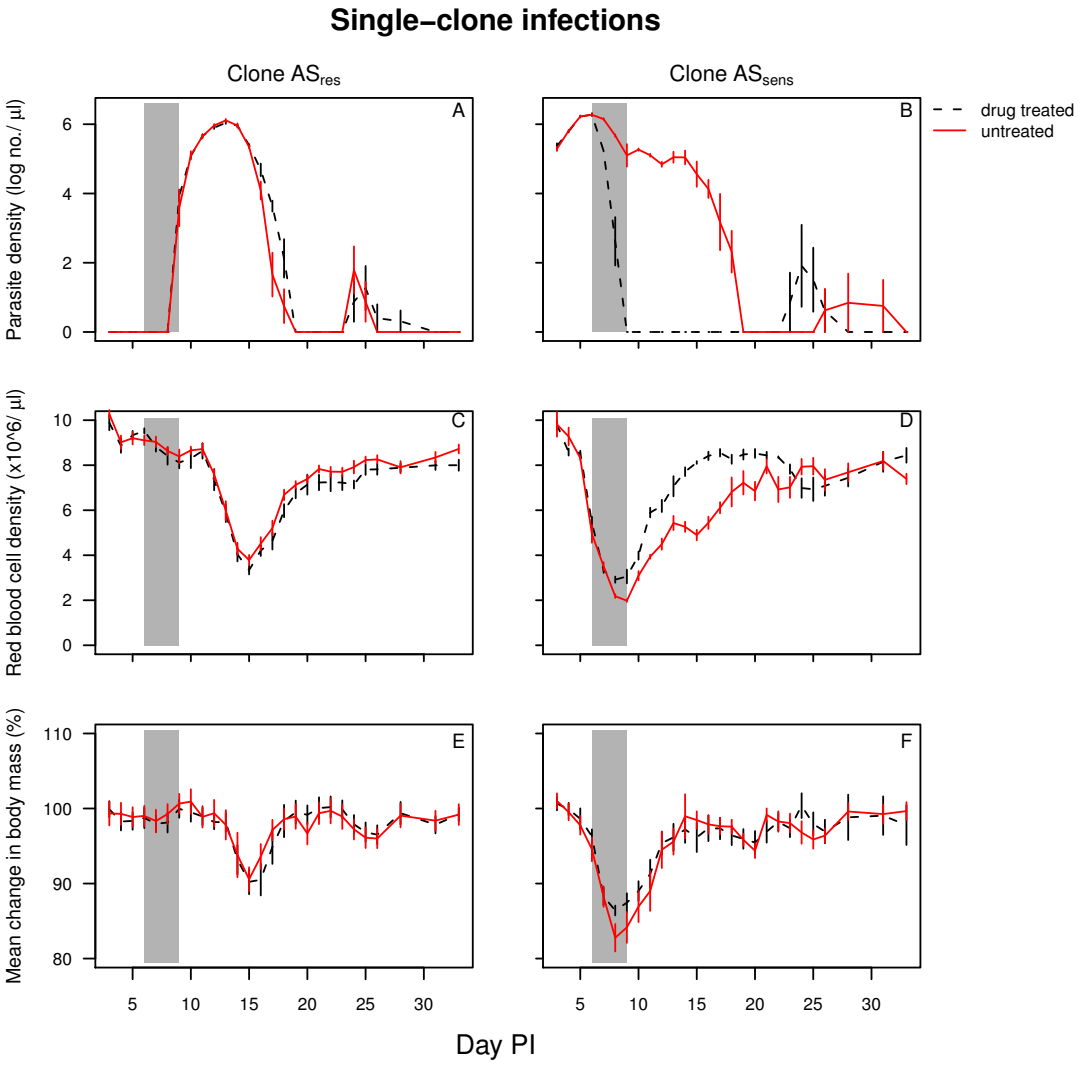

Figure 4.1 Mean (± standard error) asexual parasite density dynamics (upper panels), red blood cell densities (middle panels) and change in body mass (bottom panels) of single-clone infections of AS_{res} (left panels) and single-clone infections of AS_{sens} (right panels) which were either drug-treated (dashed black lines) or untreated (solid red lines). Drug treatments were given on days 6-9 post-infection as indicated by the grey shaded area. Inocula of clone AS_{res} consisted of 10^1 parasites, inocula of clone AS_{sens} of 10^6 parasites. Data are means of up to nine mice (Table 4.1).

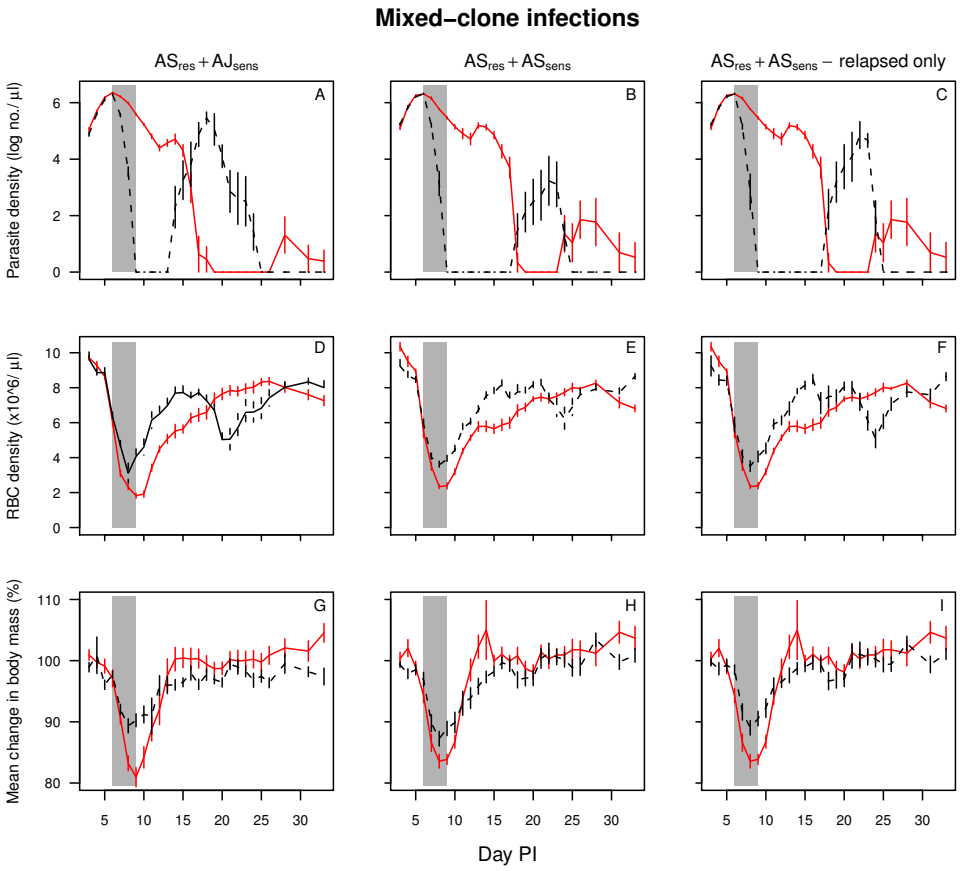

Figure 4.2 Mean (± standard error) parasite dynamics (upper row), red blood cell density (middle row) and weight (bottom row) of mixed infections AS_{res} + AJ_{sens} (left column) and AS_{res} + AS_{sens} (middle and right columns). The middle column shows AS_{res} + AS_{sens} infections with all mice included, the right column shows AS_{res} + AS_{sens} infections where the three mice that did not relapse were excluded. Infections were either drug-treated (black dashed lines) or left untreated (red solid lines). Drug treatment was given on days 6-9 post-infection, as indicated by the gray shaded area. Data are means of up to nine mice (Table 4.1).

Mixed infections of AS_{res} + AS_{sens} also resulted in a pronounced parasite relapse, although only observed in 6 out of 9 infections (Figure 4.2bc, 4.3). In those six infections, the parasite densities at peak relapse did not differ significantly from the relapses seen for AS_{res} + AJ_{sens} infections (*clone*: $F_{1,10}$=3.5, p=0.092), but it occurred 3.5 days later (*clone*: $F_{1,10}$=45, p<0.001). Overall, more parasites were produced in the relapse of AS_{res} + AJ_{sens} than in the relapse of AS_{res} + AS_{sens} (Figure 4.4, *clone*: $F_{1,10}$=11, p=0.008).

Two out of the five drug-treated single-clone infections of clone AS_{sens} also relapsed (Figure 4.3a). These relapses were later than the relapse following mixed infections of AS_{res} + AS_{sens} (*competition*: $F_{1,6}$=19, p=0.005), but had similar peak parasite densities

(*competition*: $F_{1,6}$=0.7, p=0.43). The total parasite density during the relapse of single-clone AS_{sens} infections was lower than in AS_{res} + AS_{sens} infections, however, this difference was not significantly different (Figure 4.4, *competition*: $F_{1,6}$=3.1, p=0.13). Untreated single-clone infections of AS_{sens} also relapsed (two out of five infections), with the relapses occurring later in the course of infection and at lower peak parasite densities (Figure 4.3a). Similarly, single-clone AS_{res} infections relapsed, both in untreated (four out of eight) and drug-treated (four out of nine) infections.

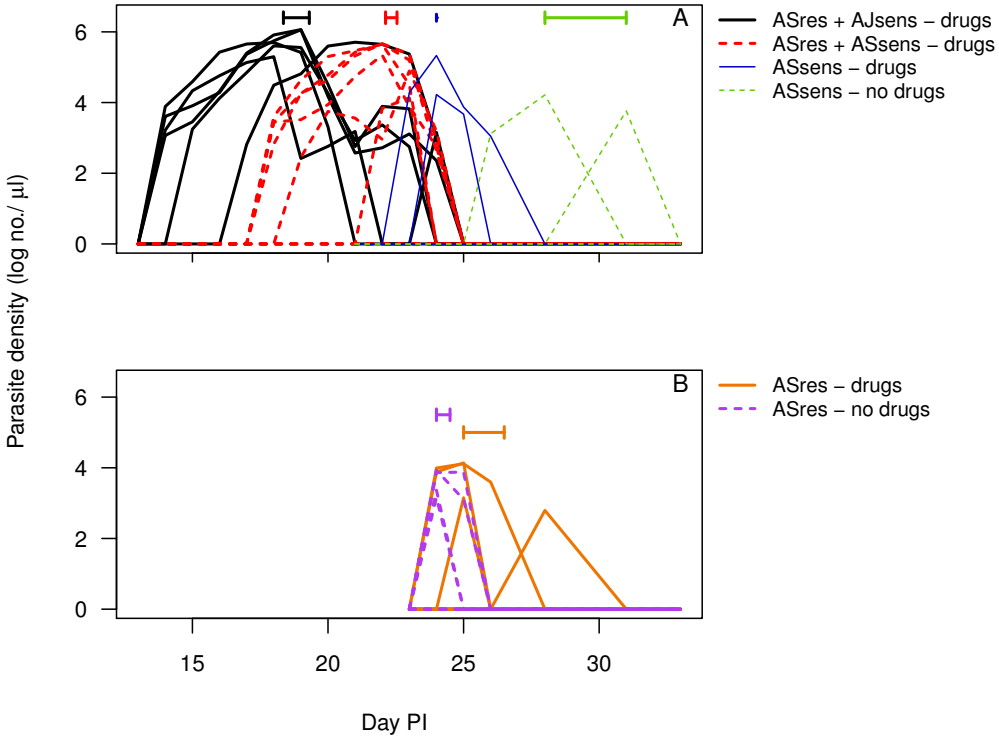

Figure 4.3 Parasite densities during relapse for drug-treated AS_{res}+AJ_{sens} (thick solid black lines), AS_{res}+AS_{sens} (thick dashed red lines), single-clone AS_{sens} (thin solid blue lines) and untreated single-clone AS_{sens} (thin dashed green lines) infections (upper panel), and parasite densities during relapse for drug-treated (orange solid line) and untreated (dashed purple line) single-clone AS_{res} infections (bottom panel). Each line represents an individual mouse. Horizontal error bars represent the lower and upper bounds of the standard error of the mean day of peak parasite density during relapse. In drug-treated mixed-clone infections of AS_{res} + AJ_{sens}, 6 out of 6 infections relapsed, in AS_{res} + AS_{sens}, 6 out of 9 infections relapsed, in drug-treated single-clone infections of AS_{sens}, 2 out of 5 relapsed and in untreated single-clone infections of AS_{sens}, 2 out of 5 infections relapsed. Of the single-clone AS_{res} infections, 4 out of 9 relapsed in drug-treated infections and 4 out of 8 relapsed in untreated infections.

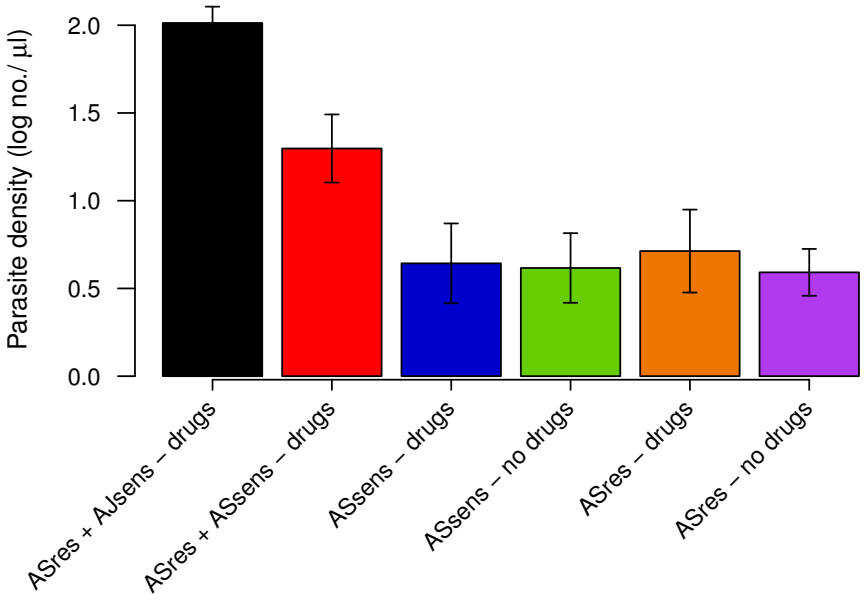

Figure 4.4 Geometric mean parasite density during relapse for drug-treated AS_{res}+AJ_{sens} (black), AS_{res}+AS_{sens} (red), single-clone AS_{sens} (blue), untreated single-clone AS_{sens} (green), drug-treated single AS_{res} (orange) and untreated AS_{res} (purple) infections. Data are geometric means of relapsed infections only (see Figure 4.3).

4.4.2 Relapse morbidity

The relapsing infections caused a second anaemic phase, both in AS_{res} + AJ_{sens} and AS_{res} + AS_{sens} infections (Figure 4.2 middle row). There was no difference observed in the maximum red blood cell loss during relapse between AS_{res} + AJ_{sens} and AS_{res} + AS_{sens} infections (*clone*: $F_{1,10}$=0.78, p=0.40), nor between AS_{res} + AS_{sens} and single-clone AS_{sens} infections (*competition*: $F_{1,6}$=2.2, p=0.19). Mice with relapsing infections did not have a distinct second period of loss in body weight (Figure 4.2 bottom row).

4.5 Discussion

This experiment shows that drug treatment of mixed infections, consisting of susceptible parasites with a low abundance of resistant parasites, leads to a pronounced parasite relapse. Similar parasite relapses have been observed previously in mixed infections of genetically distinct parasites (Wargo et al. 2007; Chapters 2,3 and 6). In this study, parasite relapses peaked at similar densities in mixed infections

of resistant and sensitive parasites from a different clonal lineage as in mixed infections with parasites from the same clonal lineage, although the peak for the latter group was later and parasite abundance during the relapse lower.

These observations are consistent with our hypothesis that drug treatment selects for rare resistant parasites in a mixed infection. With current molecular techniques, the clone-composition of the relapses in mixed infections with two clones from the same clonal lineage could not be accurately quantified; however, quantitative PCR of the mixed infections from genetically distinct clonal lineages showed that the majority of parasites present in the relapse were drug-resistant parasites. Based on the similarity in the observed parasite kinetics between unrelated and related mixed infections, it is very likely that the relapses in the mixed infections of genetically similar parasites were also predominantly caused by the drug-resistant parasites. Interestingly, however, the relapse in mixed infections of genetically related parasites occurred 3.5 days later in time and had a lower parasite density during relapse than genetically related mixed infections (Figure 4.3 and 4.4 respectively). Moreover, only six out of nine infections relapsed in the genetically related mixed infection as opposed to all infections in the genetically unrelated mixed infection. These observations suggest different underlying within-host dynamics in the genetically related versus genetically unrelated mixed infections. An explanation for this could be a lower parasite density at the time of release (Chapter 3). This is consistent with the theory that resistant parasites from the same clonal lineage could be more suppressed than resistant parasites competing with genetically distinct parasites due to niche overlap in the former. This could also explain why only six out of nine infections relapsed in these genetically similar mixed infections, opposed to all of the genetically distinct mixed infections: in the cases where resistant parasites did not relapse, competitive exclusion could have eliminated the resistant parasites. An alternative hypothesis for the later parasite peak is the involvement of the immune system, whereby a clone-specific immune system could suppress genetically similar parasites more effectively than genetically distinct parasites. However, if such cross-immunity between the genetically similar resistant and sensitive parasites exists, relapsed parasites would perhaps be expected to have a lower peak parasite, which was not observed. Moreover, similar rates of parasite increase during relapse were observed between the genetically related and unrelated infections, which does not indicate increased suppression.

All treatment groups had at least two infections that showed relapsing parasites. The difficulty in this experiment is to separate these background relapses from relapses due to competitive release of resistant parasites. The relapses of untreated AS_{sens} parasites are noticeably later and of lower parasite density than the relapses of drug-treated AS_{sens} parasites and are therefore likely based on different within-host kinetics. One explanation, which is in line with our hypothesis, is that the relapses observed in drug-treated AS_{sens} infections are initial up-selected *de novo* resistant mutants: this is possible as resistance mutations against pyrimethamine are easily selected for in a single mouse (Walliker et al. 1975). The later peak in parasite density could be explained by the low number of parasites at the time of release, as would be expected if *de novo* resistant mutants are the cause of the relapse. Under this hypothesis, it is possible that the relapse observed in the mixed infections of AS_{sens} and AS_{res} is also (partly) made up from resistant mutants, as could be the case for relapsing AJ_{sens} parasites.

This study makes use of a 'seeding' approach by inoculating a low number of parasites from the same clonal lineage to mimic *de novo* resistance mutations. Both parasite clones from the same clonal lineages were passaged 25 times since they diverged. Serial passage of *P. chabaudi* in the absence of a mosquito vector increases the virulence of the clonal lineage (Mackinnon and Read 1999) and serial passage of drug-resistant lines will likely generate compensatory mutations for resistant parasites to increase their virulence (Walliker et al. 2005). Therefore, the two clonal lineages likely have genetic differences other than single resistance-associated mutations.

Given what we know now from this experiment, a more ideal experiment would be to make use of pyrosequencing (Cheesman et al. 2007) or proportional-sequencing (Hunt et al. 2005) to determine the clone-composition in parasite relapses. These techniques can not reliably quantify genotypes lower than 5% in a mixed infection; however, even though the resistant parasites started the infection at 0.001%, the relative frequency during relapse increased well above this detection threshold of 5%. Pyrosequencing or proportional sequencing would thus be an informative tool to determine the genotypic composition during relapse. Additionally, next-generation sequencing methods, for instance Roche/454 pyrosequencing, can provide increased sensitivity though at

greater cost (Mardis 2008). A further recommendation for future work is to alter the seeding approach to more closely mimic *de novo* resistant mutants. While seeding 10 resistant parasites in the initial inoculum of 10^6 susceptible parasites represents a density of resistant parasites five orders of magnitude lower than susceptible parasites, it still results in ~15.000 parasites at the time of treatment. If the seeding event takes place just before peak parasitaemia (when, due to high parasite abundance, the likelihood of resistant mutants to arise is maximal) as opposed to a simultaneous inoculation, the study would better mimic a *de novo* resistance mutational events. Nevertheless, the best way to study the effect of drug treatment on the selection for *de novo* resistant mutants is to study *de novo* mutations themselves. With such an experiment, the effect of different classes of drugs and the importance of stepwise resistance can be established, at least in this rodent malaria model. Determining the effect of drug treatment on the up-selection of *de novo* resistance mutations is important since at present, antimalarial treatment regimes are based on the conventional wisdom that radical, high dose, drug treatment reduces the chances of *de novo* resistance to arise (WHO 2006). If this empirically unverified assumption is wrong, current treatment guidelines may need to be revised or, at the very least, the resistance management rhetoric surrounding radical parasitological cure be abandoned.

5. Competitive suppression and release of drug-resistant malaria parasites in multi-genotype infections

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5.1 Abstract

Multi-genotype malaria infections are common. The within-host dynamics of malaria parasites is thought to play an important role in the spread of drug resistance. In the absence of treatment, resistant parasites can be competitively suppressed by co-infecting susceptible parasite strains. However, drug treatment can greatly increase the relative fitness of resistant parasites because of (i) a survival advantage and (ii) competitive release as a result of reduced competitive suppression. It is thought that in the absence of treatment, an increasing number of co-infecting susceptible parasite strains results in increased competitive suppression and hence increased competitive release following drug treatment. Here, we test this hypothesis using the *Plasmodium chabaudi* mouse malaria model. We found no evidence for increased suppression nor increased competitive release with a higher number of co-infecting parasite clones. Therefore, clone multiplicity in malaria infections may have less of a significant role in the spread of drug resistance then initially assumed.

5.2 Introduction

Multi-genotype malaria infections are common (e.g. Arnot 1998; Babiker et al. 1999; Smith et al. 1999; Bruce et al. 2000; Jafari et al. 2004; A-Elbasit et al. 2007; Nwakanma et al. 2008; Vafa et al. 2008; Baruah et al. 2009; Soulama et al. 2009). The within-host dynamics of malaria parasites likely plays an important role in the spread of drug

resistance (Hastings 2003; Read and Huijben 2009), since crowding of co-infecting parasite strains[2] can lead to between-genotype competition. Direct experimental evidence on competition between co-infecting *Plasmodium falciparum* genotypes, particularly with drug-resistant parasites, cannot be ethically obtained from human infections. However, field data showing suppressed population densities of a genotype when other genotypes are present strongly supports the presence of between-genotype competition within malaria-infected hosts (Daubersies et al. 1996; Mercereau-Puijalon 1996; Smith et al. 1999; Bruce et al. 2000; Hastings 2003; Talisuna et al. 2006; Bousema et al. 2008; Harrington et al. 2009). Additionally, there is considerable direct experimental evidence for crowding in rodent malaria models (e.g. Jarra and Brown 1985; Taylor et al. 1997; de Roode et al. 2004; de Roode et al. 2005b; Bell et al. 2006) and, in particular, competitive suppression of drug-resistant *P. chabaudi* parasites in a mixed infections with drug-sensitive parasites (de Roode et al. 2004; this thesis; Wargo et al. 2007; Huijben et al. *submitted*). When mixed infections of drug-resistant and sensitive parasites are drug-treated, the relative fitness of the resistant parasites increases not only as a result of a survival advantage in the drug environment, but also by the removal of the susceptible competitors, which leads to an expansion of drug-resistant parasites. Such drug-induced competitive release was demonstrated in a two-genotype rodent malaria infection following both prophylactic (de Roode et al. 2004) and therapeutic (Wargo et al. 2007; Huijben et al. *submitted; Chapters 3 and 6*) drug treatment, which likely results in a great fitness advantage for resistant parasites.

All previous experiments on competitive release were carried out with a resistant clone competing with one susceptible genotype. In reality, resistant parasites often find themselves in infections with many more susceptible genotypes. Multi-clone infections can arise in two different ways: by a multi-clone infection from a single mosquito (co-infection; Nwakanma et al. 2008; Mohanty et al. 2009) or from two sequential infections from mosquitoes infected with a single (or multi-) clone (super-

[2] The terminology of parasite strains, genotype and clone is tricky. When considering *Plasmodium falciparum* infections, parasite strain and genotype are used interchangeably, indicating genetically similar parasites derived from the same ancestor. This term is ambiguous since recombination within the mosquito results in great genetic variety. When considering *P. chabaudi* parasites, parasite clone is used to describe parasites originating from the same clonal lineage. Due to multiple passages within a clonal lineage and mutation accumulation, different parasites from the same clone may have some genetic variation and are thus not 'true' clones.

infection; de Roode et al. 2005a). In highly endemic areas such as large parts of sub-Saharan Africa, an entomological inoculation rate (EIR) of several hundred infective bites per year have been reported (Trape and Rogier 1996; Beier et al. 1999; Smith et al. 2005; Gemperli et al. 2006), which leads to high levels of multi-genotype infections. The number of parasite strains observed within a person range from one to more than five, sometimes exceeding ten strains per infection (e.g. Babiker et al. 1999; Konate et al. 1999; Beck et al. 2001; Magesa et al. 2002; Sutherland et al. 2002; Schoepflin et al. 2009). Competition for resources in such multi-genotype infections is likely strong since host resources have to be shared among multiple parasite strains (Hastings and D'Alessandro 2000; de Roode et al. 2003). Consider for example a situation whereby either two clones or five clones are competing within the host, with one of the clones being resistant and the host resources are equally shared among the clones. In the two-clone example, removal of the susceptible parasites may lead to a doubling of resistant parasites, but in the five-clone example the removal of the four susceptible clones may lead to a 500% increase in resistant parasites. To the best of our knowledge, there is no empirical data on the effect multi-genotype competition has on drug-resistant parasites in the absence and presence of drug treatment. We hypothesize that resistant clones are increasingly suppressed with an increasing number of susceptible genotypes co-infecting the host, while drug treatment likely increases the competitive release, with a greater relative release when strong suppression occurs (Hastings and D'Alessandro 2000).

The aim of this study was to evaluate the within-host dynamics in multi-genotype infections, with a specific focus on resistant parasites in the presence and absence of drug treatment. Using a *P. chabaudi* mouse model, up to four genetically distinct clonal lineages in mixed infections were quantified through time. Strong competitive suppression was observed for resistant parasites competing with each of three other susceptible clonal lineages. However, we found no evidence that the extent of competitive suppression or competitive release is affected by the number of co-infecting clones.

5.3 Material and methods

5.3.1 Parasites and hosts

In this experiment, four genetically distinct *P. chabaudi* clones were used: drug-resistant clone $AS_{8p(pyr\text{-}1A)}$ (hereafter referred to as clone R), and drug-sensitive clones AJ_{8p}, AT_{2p} and CB_{2p} (hereafter referred to as clones AJ, AT and CB respectively) . All clones were originally isolated from thicket rats and subsequently cloned (Beale et al. 1978). Clone R was made resistant in the laboratory by exposure to a high dose of pyrimethamine in a single passage (Walliker et al. 1975). Female C57Bl/6 laboratory mice (Charles River Laboratories), aged approximately 10 weeks, were used as hosts. All mice received 0.05% PABA-supplemented drinking water to enhance parasite growth (Jacobs 1964). The mice were fed on Laboratory Rodent Diet 5001 (LabDiet, PMI Nutrition International) and were kept on a 12:12 L:D cycle.

5.3.2 Experimental design, infections and drug treatment

Mice were infected with either a single-clone infection of clone R, or a mixed infection with clone R and one, two or all three of the other clones. All possible combinations of clones co-infecting clone R were included in the study. Half of the mice were drug-treated, which came to a total of 16 different treatment groups (Table 5.1). Each experimental treatment consisted of 5 mice, except for the untreated infections with three and four clones. These groups consisted of respectively 7 and 9 mice, since we expected a higher mortality to occur in infections with a higher clone multiplicity. The mice of treatment groups of 5 mice were housed in one cage, mice of treatment groups of 7 and 9 mice were housed in two cages.

Table 5.1 Experimental set-up. All mice in each treatment group were inoculated with clone R and none, one, two or all of the clones AJ, AT and CB. An 'x' in the column of each of the respective clones indicates the inclusion of that clone in the inoculum. The column 'n' specifies the number of mice that were included in the treatment group at the start of the experiment with the number that survived in brackets. Half of the treatment groups received drug treatment, which was administered on days 6-9 post-infection.

Treatment	R	AJ	AT	CB	n
No drug treatment					
R	x				5 (5)
R-AJ	x	x			5 (5)
R-AT	x		x		5 (5)
R-CB	x			x	5 (2)
R-AJ-AT	x	x	x		7 (6)
R-AJ-CB	x	x		x	7 (3)
R-AT-CB	x		x	x	7 (5)
R-AJ-AT-CB	x	x	x	x	9 (5)
Drug-treated					
R	x				5 (5)
R-AJ	x	x			5 (5)
R-AT	x		x		5 (5)
R-CB	x			x	5 (5)
R-AJ-AT	x	x	x		5 (5)
R-AJ-CB	x	x		x	5 (5)
R-AT-CB	x		x	x	5 (5)
R-AJ-AT-CB	x	x	x	x	5 (5)

Infections were initiated with an intraperitoneal injection of 10^6 parasites of each clone. Multi-clone infections were established by mixing the different parasite clones in the inoculum, so that only a single inoculation was necessary. In multi-clone infections, each clone was initiated with 10^6 parasites, so that the four-clone infection received a four fold higher parasite dose than the single-clone R infection. We did this because analysing competition requires comparison of the performance of an individual clone in the presence and absence of competition, starting from the same initial parasite dose. Up to a four fold higher dose of 10^6 parasites has little effect on overall parasite dynamics or host health (Timms et al. 2001).

Drug treatment was initiated on day 6 post-infection (PI), when parasite-induced weight loss and anaemia became pronounced, and subsequently repeated on days 7 to 9. Drug treatment consisted of 8 mg pyrimethamine/kg mouse weight dissolved in dimethyl sulfoxide (DMSO) and was administrated by intraperitoneal injection of 50 µl. Untreated controls were given DMSO-only.

5.3.3 Monitoring of infections

Mice were monitored daily from day 3 to day 21 PI and three times a week up to day 35 PI. During sampling, mouse weight (to the nearest 0.1 gram) and red blood cell density using flow-cytometry (Beckman Coulter) were measured, and a thin blood smear was taken. Additionally, 5 µl of blood was taken for counting the total parasite density and 10 µl was taken for counting gametocyte density, both by using quantitative PCR.

DNA was extracted from 5 µl of blood, which was performed on the ABI Prism® 6100 Nucleic Acid PrepStation according to manufacturer's instructions. RNA was extracted from 10 µl of blood, using the 'RNA Blood-DNA' method on the ABI Prism® 6100 Nucleic Acid PrepStation. RNA was converted to single stranded cDNA immediately after extraction, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Wargo et al. 2006). Both DNA and cDNA were stored at -80°C until quantification.

To measure parasite density (asexual parasites and gametocytes combined) of each clone, quantitative PCR was performed on extracted DNA using clone-specific assays. Clone R and AJ were measured using the R-specific and AJ-specific *PcCG1* assay respectively, as described in Drew and Reece (2007). Clone AT was quantified using the *msp-1 AT* assay and clone CB was quantified using the *msp-1 CB* assay, both described in Bell et al. (2006). We only quantified the gametocyte density of clone R, since this was our focal clone and no clone-specific gametocyte qPCR assay is yet developed for clones AT and CB. For gametocyte quantification, cDNA was used instead of DNA on the R-specific *PcCG1* assay. Due to loss of samples, gametocyte data is only available from day 3 to day 17.

The PCR reactions for the R-specific and AJ-specific *CG1*-assays had a total volume of 25 µl, which consisted of 7 µl DNA or cDNA (clone R only), 900 nM forward and reverse clone-specific primers, 250 nM TaqMan® MGB *PcCG1* probe (Applied Biosystems) and 1x PerfeCTa™ qPCR FastMix™ (Quanta Biosciences). The reactions of the *msp-1 AT* assay and msp-*1 CB* assay both also had a total volume of 25 µl, these consisted of 2 µl DNA, 300 nM forward and reverse clone-specific primers and 200 nM TaqMan® MGB *msp-1* probe. All reactions were run on the ABI Prism® 7500 Fast

System, using the assay: 95°C for 2 minutes, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Quantification was based on serial dilutions of clone-specific DNA and cDNA standards of known total parasite and gametocyte density, determined beforehand by microscopy (Cheesman et al. 2003). Parasite density for clone R was determined for days 3-35, for clones AJ, AT and CB up to day 23.

5.3.4 Statistical analysis

Analysis was done using analysis of variance in R 2.9.0 (R Development Core Team 2009). For analysis of competition in untreated infections, the geometric mean parasite density was calculated over days 3-35 PI for clone R and over days 3-23 PI for clones AJ, AT and CB, which was the monitoring period of the susceptible clones. For analysis of drug-treated infections, geometric mean parasite density of clone R post-treatment was calculated (day 7-35 PI). The total parasite density of all parasite clones combined in the infection was estimated by summing the parasite densities of each infecting clone in mixed infections up to day 23. As a measure of transmission potential, the predicted infectiousness was calculated for clone R after the start of treatment until day 17 using gametocyte densities in the density-infectivity function q1 as described in the general introduction of this thesis. As a measurement of virulence, the mean weight and mean red blood cell density during the infection was calculated, as well as the maximum weight loss and maximum red blood cell loss for a measurement of virulence during the acute infection.

As explanatory variables, *drug treatment* (drug-treated/untreated) and *co-infecting clones* (AJ/AT/CB/AJ-AT/AJ-CB/AT-CB/AJ-AT-CB) or *number of co-infecting clones* (0/1/2/3) were included. Maximal models were fitted first and non-significant interactions were removed to obtain minimal models.

Many treatment groups had one or more mice that appeared to have received a lower inoculum of parasites than intended, seen as a lag in growth of the parasites of a day or more (Chapter 3). Since the different clones were mixed into a single inoculum, the mice that received a lower dose of parasites received an overall lower dose of all parasite-clones, but the parasite ratio was maintained. Parasite dynamics in individual mice are given in figures A1 and A2 in the appendix to this chapter. To account for any effect of the lower inocula, the R parasite density at the first sampling day (day 3) was

included in the models as a covariate. This factor did not have a significant effect in any of the tests was therefore eliminated from the models.

A total of 14 mice died or were euthanized during the course of the infection, all of them in untreated groups (Table 5.1). All deaths occurred between days 8 and 13 post-infection, with a peak on day 10. These 14 mice were excluded from the analyses.

5.4 Results

5.4.1 Asexual parasite dynamics

In untreated infections, all parasite clones reached peak parasite density around day 6 or 7, after which clones AJ, AT and CB persisted at higher parasite densities than clone R (Figure 5.1, Figure A1 – appendix to this chapter). When the infections were drug-treated, the susceptible parasite clones dropped in parasite density and were below or around detection threshold around day 12 post-infection, at which time clone R increased in parasite abundance with distinct second peaks in R parasite density observed when in competition with two or more susceptible clones (Figure 5.2, Figure A2 – appendix to this chapter). In the absence of drug treatment, the resistant parasites were competitively suppressed by all three clones, which are known to be more virulent than clone R (Figure 5.3a, *presence of competitors*: $F_{1,34}=61$, $p<0.001$) (Bell et al. 2006), while drug treatment resulted in clone R performing as good or better than in the absence of competition (Figure 5.3b). Taking the different clones together, no differences were observed in the strength of suppression between the number of co-infecting clones (Figure 5.4 – untreated infections, *number co-infecting clones*: $F_{2,28}=0.1$, $p=0.89$). In drug-treated infections, the resistant parasites were released following treatment. In competition with multiple clonal genotypes, the resistant parasites had lower densities before being released, but were released to a higher density than the resistant parasites in the absence of competition or when competing with one clone (Figure 5.3b). Therefore, over the entire infection period following drug treatment, the parasite density of clone R post-treatment was similar between treatments with different number of co-infecting susceptible clones (Figure 5.4, *no. of co-infecting clones*: $F_{2,32}=0.46$, $p=0.64$).

Few differences were found in the effect of the different susceptible clones on the parasite dynamics of clone R. The strength of competitive suppression in untreated infections was less strong with a co-infection with clone CB than with AJ or AT (Figure 5.5a, *clone*: $F_{2,9}$=6.4, p=0.019), similarly, the combination AJ + CB exerted less competitive suppression than the other two combinations (Figure 5.5b – $F_{2,11}$=7.4, p=0.009). Clone R in competition with one other clone followed similar parasite dynamics following drug treatment as in the absence of treatment. The genotype of the co-infecting clone did not affect the extent of competitive release (Figure 5.5, one co-infecting clone: $F_{2,12}$=0.04, p=0.97; two co-infecting clones: $F_{2,12}$=0.55, p=0.59 respectively).

Since no single-clone infection of the susceptible parasite clones were included in this experiment, competitive suppression could not be studied for these clonal lineages. However, a comparison could be made between with co-infections with clone R only, and with one or two of the other, virulent, clones. A co-infection with clone R only has been shown before not to affect the performance of AJ or AT, and only slightly suppress CB (Bell et al. 2006). Strikingly, clone AJ performed better in competition with one additional virulent clone (AT or CB), or both together (AT plus CB), than with just clone R (Figure 5.6a, p_{adj}=0.013, p_{adj}=0.002 respectively). Clones AT and CB performed as well with just clone R as with 1 or 2 of the other clones (Figure 5.6bc, p=0.28, p=0.08 respectively). Thus, each of the susceptible clones had similar or more parasite densities when sharing the host with only clone R or with one or two additional virulent parasite clones.

As a consequence, in untreated infections with an increasing number of co-infecting clones, the total parasite density increased as well (Figure 5.7, *number of infecting clones*: $F_{3,32}$=50, p<0.001). Overall, drug-treated infections had a lower total parasite density than untreated infections (*drugs*: $F_{1,68}$=117, p<0.001). The total parasite densities in drug-treated infections were the same for double, triple or quadruple infected mice (*no. of co-infecting clones*: $F_{2,32}$=2.5, p=0.10).

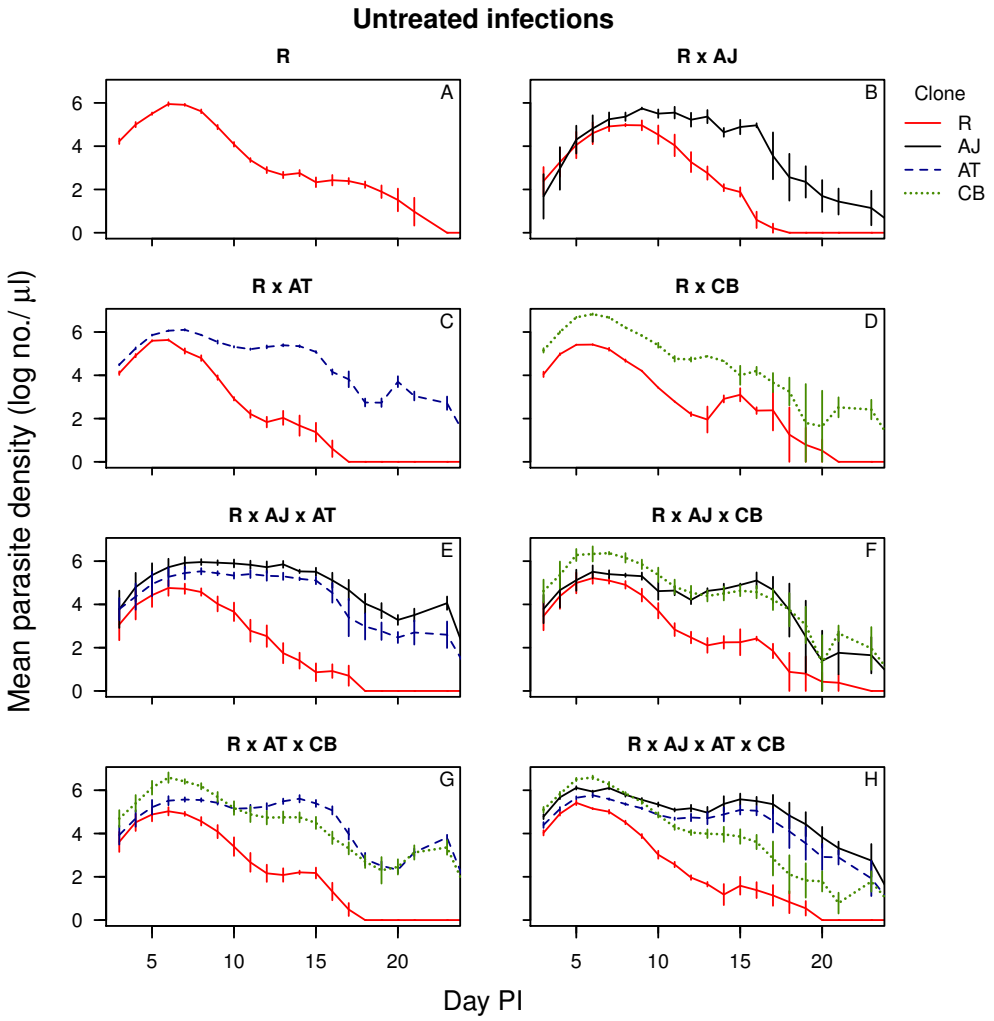

Figure 5.1 Parasite dynamics of untreated infections of clone(s): R alone (a), R with AJ (b), R with AT (c), R with CB (d), R with AJ and AT (e), R with AJ and CB (f), R with AT and CB (g), R with AJ, AT and CB (h). The total parasite densities for clone R are shown in solid red line, for clone AJ in solid black line, for clone AT in dashed blue line and for clone CB in dotted green line. Data are means (± standard error) of up to 6 mice (table 5.1). The mixed infection of R with CB consisted of only two surviving mice.

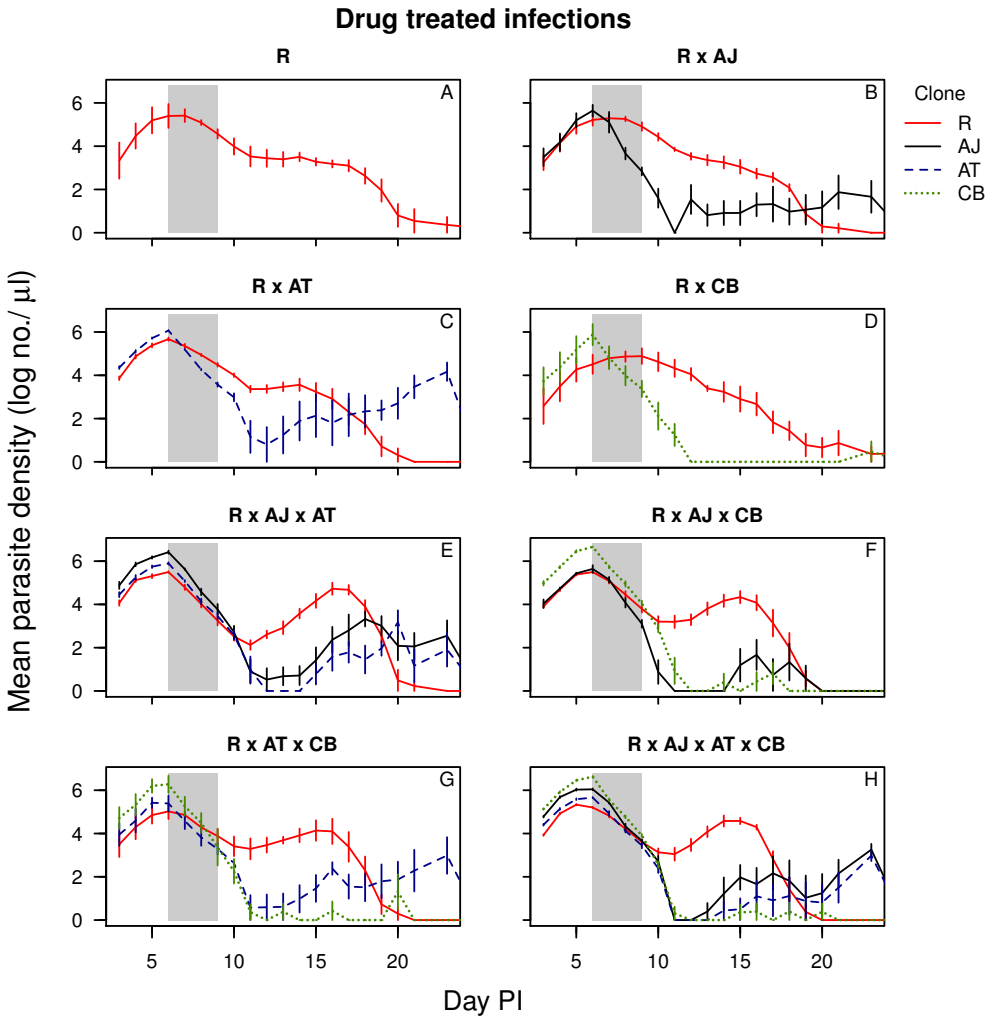

Figure 5.2 Parasite dynamics of drug-treated infections of clone(s): R alone (a), R with AJ (b), R with AT (c), R with CB (d), R with AJ and AT (e), R with AJ and CB (f), R with AT and CB (g), R with AJ, AT and CB (h). The total parasite densities for clone R are shown in solid red line, for clone AJ in solid black line, for clone AT in dashed blue line and for clone CB in dotted green line. Data are means (± standard error) of 5 mice (table 5.1). Drug treatment was given on days 6-9 post-infection, as indicated by the shaded area.

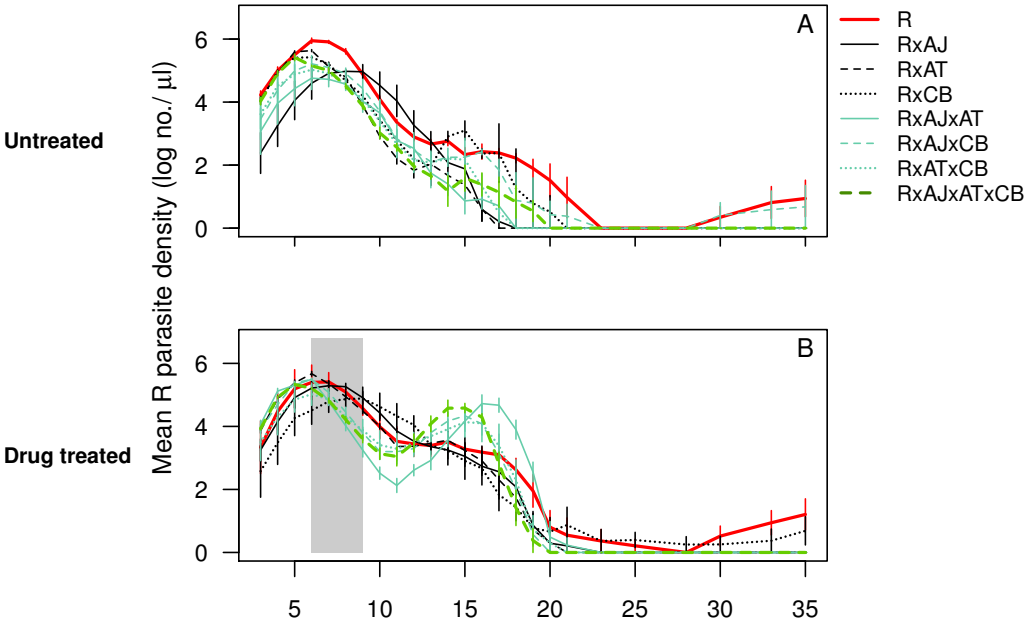

Figure 5.3 Parasite dynamics of clone R in untreated (a) and drug-treated (b) infections of clone R alone (solid thick red line), R with AJ (solid black line), R with AT (dashed black line), R with CB (dotted black line), R with AJ and AT (solid blue line), R with AJ and CB (dashed blue line), R with AT and CB (dotted blue line) and R with AJ, AT and CB (thick dashed green line). Drug treatment was given on days 6-9 post-infection, as indicated by the shaded area. Data are means (± standard error) of up to six mice (Table 5.1). The mixed infection of R with CB consisted of only two surviving mice.

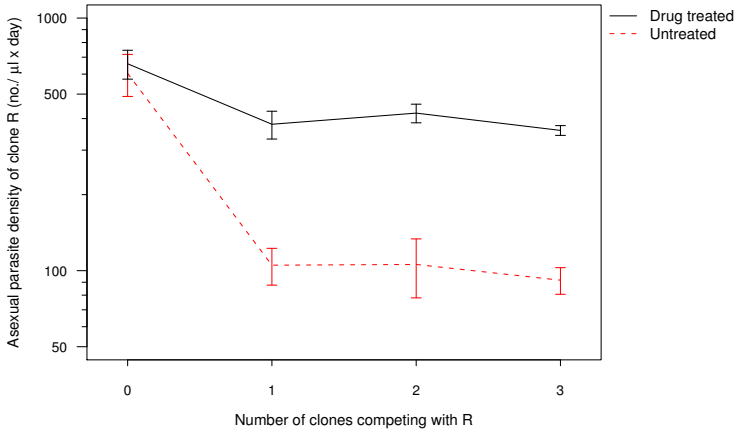

Figure 5.4 Geometric mean daily parasite density of clone R in drug-treated (solid black line) and untreated (dashed red line) infections of either clone R alone (0 competing clones) or in a co-infection with 1, 2 or 3 other clones. Data are means (± standard error). The means for 1 and 2 competing clones have been derived by combining the data from these respective treatments.

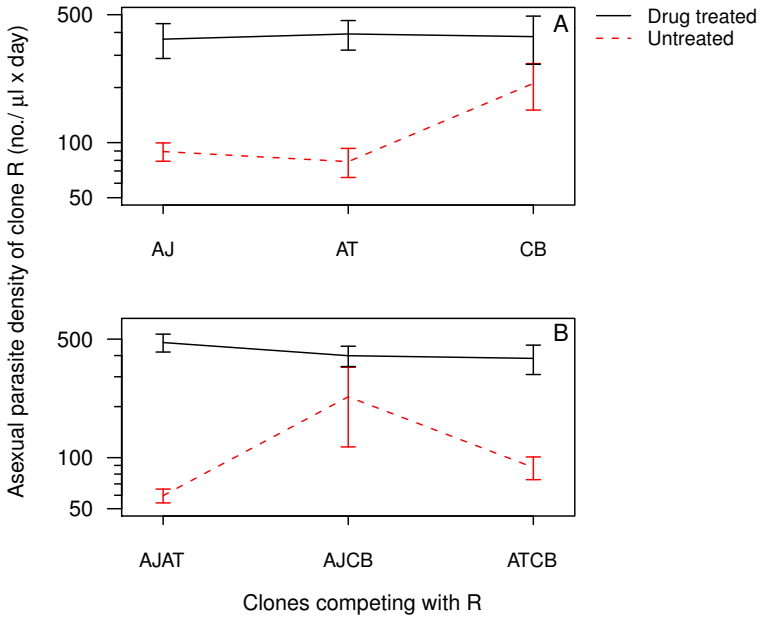

Figure 5.5 Geometric mean daily parasite density of clone R in drug-treated (solid black line) and untreated (dashed red line) infections, when competing with one other clone (AJ, AT, or CB, upper graph), or two other clones (AJ and AT, AJ and CB, or AT and CB; lower graph). Data are means (± standard error) of up to six mice. The mixed infection with CB only consisted of only two surviving mice.

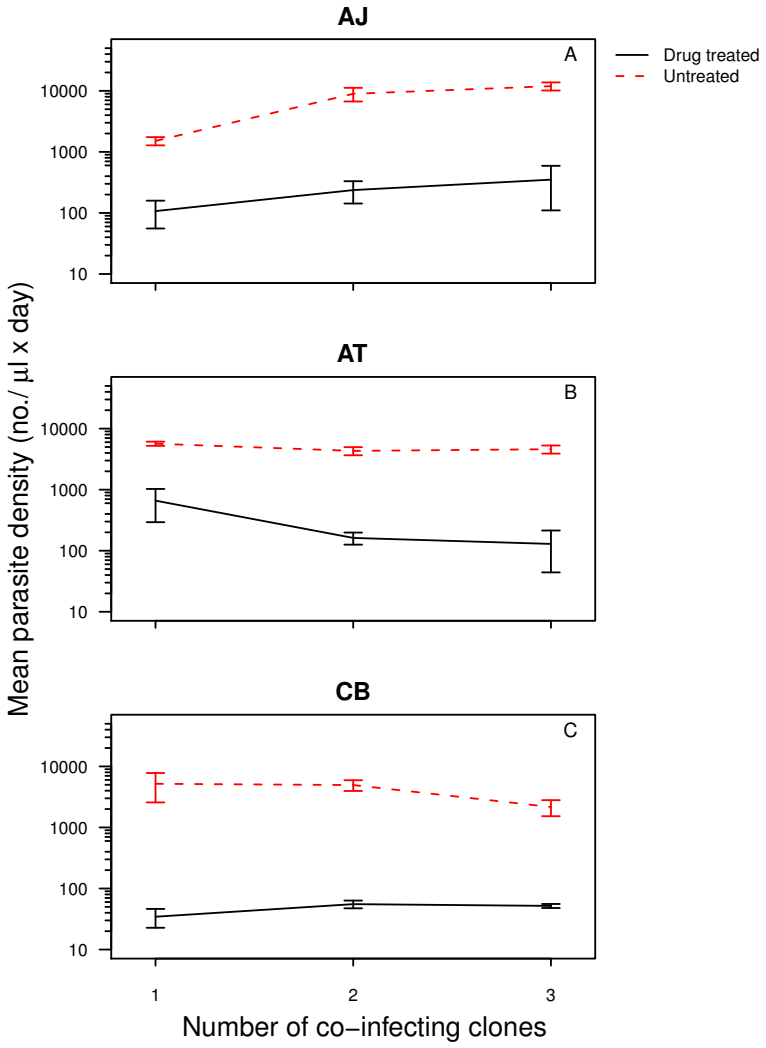

Figure 5.6 Geometric mean daily parasite density for non-focal clones AJ (a), AT (b) and CB (c) in drug-treated (solid black line) and untreated (dashed red line) infections, when competing with only one clone (less virulent clone R), with two clones (clone R with one of the other virulent clones) or with three clones (clone R with both other virulent clones). Data are means (± standard error). The means for 2 co-infecting clones have been derived by combining the data from the two different treatment groups.

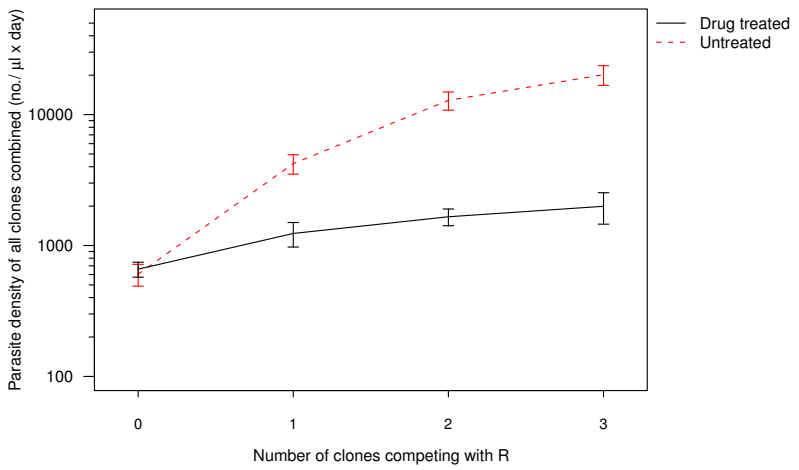

Figure 5.7 Geometric mean daily total parasite density of all clones combined in drug-treated (solid black line) and untreated (dashed red line) infections of either clone R alone (0 competing clones) or in a co-infection with 1, 2 or 3 other clones from day 3 to 23 PI. Data are means (± standard error). The means for 1 and 2 competing clones have derived by combining the data from these respective treatments.

5.4.2 Transmission potential

The gametocytes of clone R peaked around day 6 in untreated infections, after which densities dropped and peaked again around day 13. Clone R produced higher gametocyte densities throughout the infection in the absence of competition. Of note is that clone R did not produce a distinct second gametocyte peak when in competition with one or more of the other parasite clones (Figure 5.8a). When drug treatment was given, all infections showed a second peak in gametocyte production (Figure 5.8b), which was similar to or higher than clone R in the absence of competition.

The predicted number of mosquitoes infected with resistant parasites was reduced when in competition (untreated infections, *presence of competitors*: $F_{1,34}$=37, p<0.001). An increasing number of co-infecting genotypes did not decrease the transmission potential of resistant parasites in untreated infections (Figure 5.9, *no. of co-infecting clones*: $F_{2,28}$=1.7, p=0.20). As in asexual parasite dynamics, drug treatment resulted in a release of resistant gametocytes which doubled their transmission potential (Figure 5.9, *drugs*: $F_{1,68}$=214, p<0.001). The number of co-infecting clones did not affect the competitive release of resistant gametocytes, such that the estimated infectivity of

clone R was comparable between infections with one, two or three co-infecting parasites (Figure 5.9, *no. of co-infecting clones*: $F_{2,32}$=2.1, p=0.14).

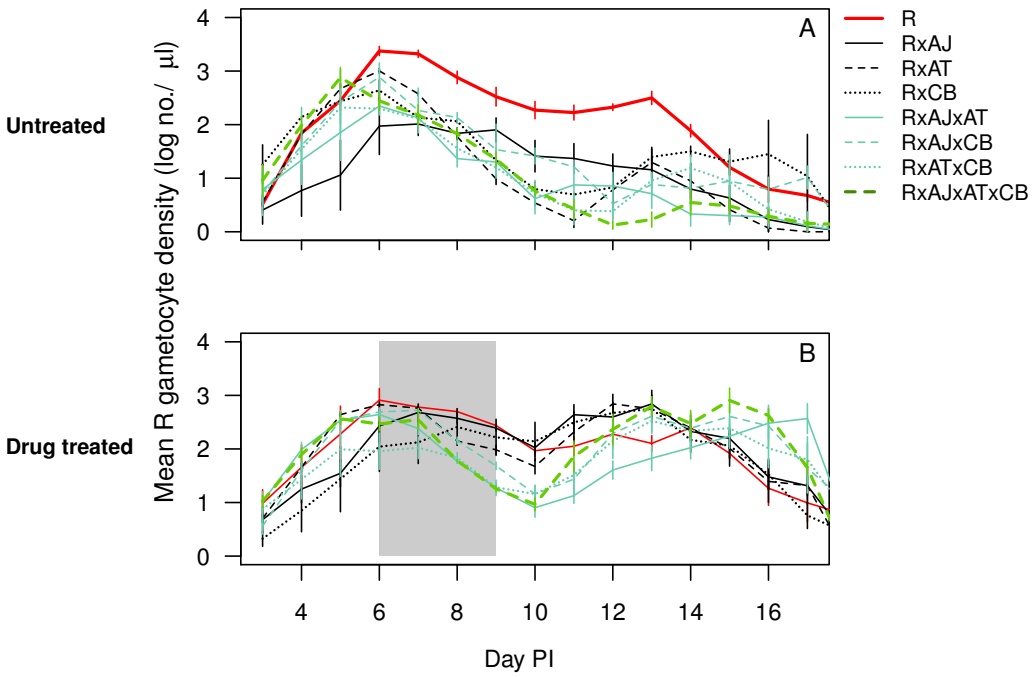

Figure 5.8 Gametocyte dynamics of clone R (day 3-17) in untreated (a) and drug-treated (b) infections of clone R alone (solid thick red line), R with AJ (solid black line), R with AT (dashed black line), R with CB (dotted black line), R with AJ and AT (solid blue line), R with AJ and CB (dashed blue line), R with AT and CB (dotted blue line) and R with AJ, AT and CB (thick dashed green line). Drug treatment was given on days 6-9 post-infection. Data are means (± standard error) of up to six mice (Table 5.1). The mixed infection of R with CB consisted of only two surviving mice.

The co-infecting genotypes seemed to affect the estimated infectiousness of clone R, with competition with clone CB resulting in slightly less competitive suppression than the other clones (Figure 5.10a, *co-infecting clone(s)*: $F_{2,9}$=4.4, p=0.047). For three-clone co-infections, the susceptible clone combination did not affect the predicted infectivity of the resistant parasites (Figure 5.10b, *co-infecting clone(s)*: $F_{2,11}$=2.0, p=0.18). In drug-treated infections, the predicted infectivity was similar between the different co-infecting clones, both in double infections (drug-treated infections, *co-infecting clone*: $F_{2,12}$=0.5, p=0.62) and triple infections (*co-infecting clone*: $F_{2,12}$=1.3, p=0.32).

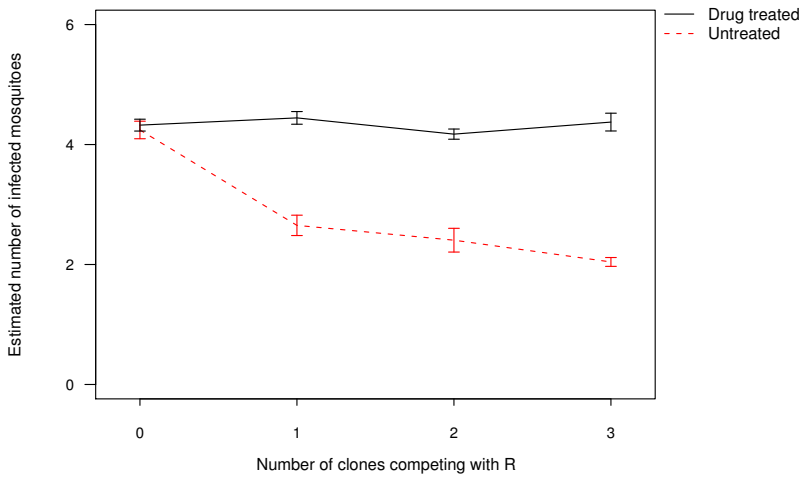

Figure 5.9 Estimated number of infected mosquitoes (out of n=100) clone R post-infection (day 7-17) in drug-treated (solid black line) and untreated (dashed red line) infections of either clone R alone (0 competing clones) or in a co-infection with 1, 2 or 3 other clones. Data are means (± standard error). The means for 1 and 2 competing clones have been derived by combining the data from these respective treatments.

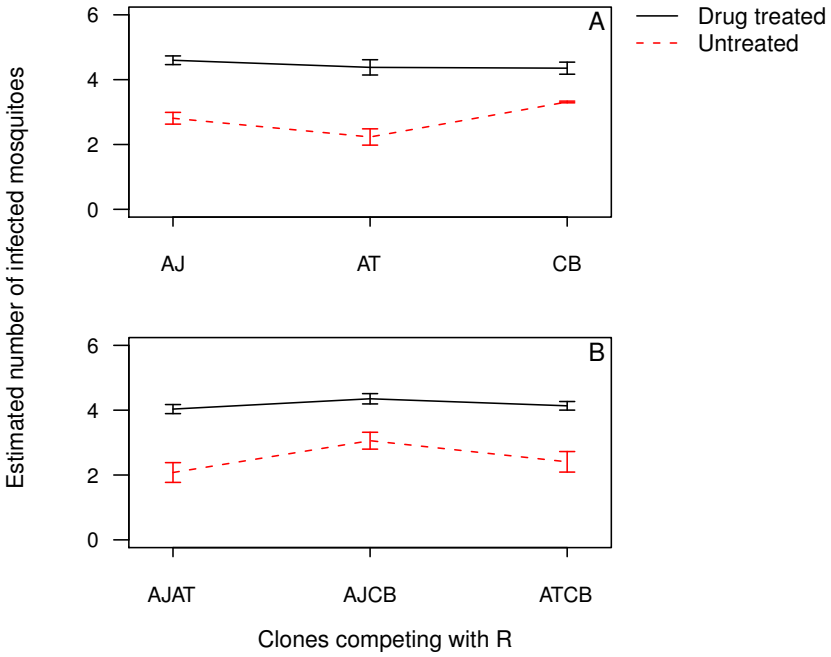

Figure 5.10 Estimated number of infected mosquitoes (out of n=100) of clone R in drug-treated (solid black line) and untreated (dashed red line) infections, when competing with one other clone (AJ, AT, or CB, upper graph), or two other clones (AJ and AT, AJ and CB, or AT and CB; lower graph). Data are means (± standard error) of up to six mice. The mixed infection with CB only consisted of only two surviving mice.

5.4.3 Host health

Untreated infections of clone R in combination with other susceptible clones caused a higher degree of anaemia during the infection than clone R in a single-clone infection (Figure 5.11a, mean red blood cell density: $F_{1,34}$=39, p<0.001). No differences were found in mean body mass of the mice (Figure 5.11b, $F_{1,34}$=0.01, p=0.92). The number of additional susceptible parasite clones in the infection did not have an effect on the mean mouse body mass (Figure 5.11b – untreated infections, $F_{2,28}$=0.24, p=0.79), nor on the maximum weight loss during the acute infection ($F_{2,28}$=0.60, p=0.56). However, mice with more clones in the infection were more anaemic on average during the infection (Figure 5.11a – untreated infections, $F_{2,28}$=12, p<0.001). This result could not be explained by the inclusion of the presence of any of the three clones as a co-variate. Increased maximum red blood cell loss during the acute infection with more additional clones was not found ($F_{2,28}$=2.4, p=0.11).

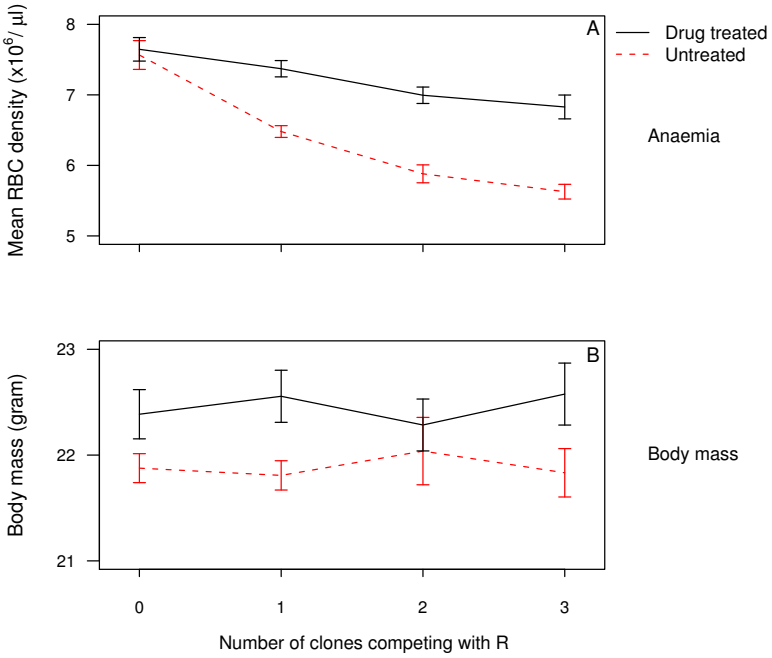

Figure 5.11 Mean daily red blood cell density (a) and body mass (b) in drug-treated (solid black line) and untreated (dashed red line) infections of either clone R alone (0 competing clones) or in a co-infection with 1, 2 or 3 other clones. Data are means (± standard error). The means for 1 and 2 competing clones have been derived by combining the data from these respective treatments.

The different susceptible genotypes of the co-infecting clonal lineages did not have a different impact on host health (*co-infecting clone*, body mass: $F_{2,9}$=0.72, p=0.51; red blood cell density: $F_{2,9}$=1.56, p=0.26). Also in triple infections, the different clone combinations did not affect host health differently (body mass: $F_{2,11}$=0.48, p=0.63; red blood cell density: $F_{2,11}$=0.31, p=0.74). The same results were found when using the maximum loss in body mass and maximum red blood cell loss during the acute infections.

Drug treatment significantly reduced disease-related morbidity, with a higher mean body mass (*drugs*: $F_{1,71}$=7.1, p=0.009) and higher mean red blood cell density ($F_{1,68}$=91, p<0.001). The same was true for maximum weight and red blood cell density loss. While drug treatment relieved the symptoms of the infection, infections with more clones were still more anaemic than infections with only clone R or clone R with one other clone (Figure 5.11a – drug-treated infections, *no. of co-infecting clones*: $F_{2,32}$=4.1, p=0.026). No such difference was observed for mean weight (Figure 5.11b – $F_{2,32}$=0.4, p=0.68).

5.5 Discussion

Multi-clone infections are common in patients with malaria in endemic areas and could result in both a reduction of the fitness of drug-resistant parasites (in the absence of treatment) and an increase of fitness by means of competitive release following drug treatment (de Roode et al. 2004; Wargo et al. 2007; Huijben et al. *submitted*). This study shows strong competitive suppression for drug-resistant parasites in untreated multi-genotype infections and competitive release when these infections were drug-treated. Unexpectedly, we found that the number of co-infecting susceptible genotypes did not affect competitive suppression, or the extent of competitive release, which suggests that resistant parasites in multi-clonal environments are not worse off than resistant parasites sharing their host with only one susceptible genotype.

Why was competition not affected by the number of co-infecting parasite clones? Competition between co-infecting malaria parasites is thought to arise from competition for resources, most dominantly red blood cell densities (Yap and

Stevenson 1994; Mideo et al. 2008; Mideo 2009), though other resources such as glucose may also play a role (de Roode et al. 2003). Immune-mediated competition, whereby antibodies produced against one parasite genotype are cross-reactive against other genotypes, is likely also a factor in competition between different malaria genotypes (Mota et al. 2001). In this latter case, the immune response provoked by one strain negatively affects a co-infecting strain.

No competitive interactions between the different drug-sensitive genotypes were observed in this experiment. Consequently, a higher total parasite density and a higher degree of anaemia during the acute infection were observed with an increasing number of parasite clones. In other words, the carrying capacity of the infection appeared to increase with increasing genotypic diversity. Since the carrying capacity of the infection increased with an increasing number of co-infecting susceptible clones, no additional competitive suppression was exerted on the drug-resistant parasites. One possible explanation for the increasing carrying capacity is that the different drug-sensitive genotypes inhabit a slightly different niche, for example by having a different red blood cell age preference (Mideo et al. 2008), which results in enhanced resource exploitation. We would expect that there is a limit to this enhanced exploitation of the host; however, with the combination of four clones in this experiment, we did not seem to have reached that limit.

Our results suggest that resistant parasites experienced no additional cost to being in an infection with more than one susceptible parasite strain and could persist in multi-genotype environments. Studies in areas of seasonal malaria have shown that resistant parasites can persist during the long dry season in mixed infections with susceptible parasites (Abdel-Muhsin et al. 2004; Ord et al. 2007; Babiker 2009) and are not competitively excluded. Such infections, however, are chronic and asymptomatic, whereas predominantly the acute phase of the infection is studied in this experiment. Competition during the chronic phase of an infection may be mediated through a different mechanism since red blood cells are less of a limiting factor. However, previous research has shown that parasite dynamics of co-infecting clones during the chronic phase of the infection are more or less independent from each other (Bell et al. 2006).

The infections in this study were initiated with a simultaneous inoculum of different parasite genotypes, mimicking a co-infection by a multi-genotype infected mosquito. However, infections that arise from multiple infectious bites, several days or weeks apart are very common as well. A turn-over of different parasite clones has been frequently observed in the field (Daubersies et al. 1996; Farnert 2008). The dynamics following such super-infections may result in different competitive outcomes, whereby competition may be more intense for genotypes that infect later on in the infection (de Roode et al. 2005a). Similarly, the experiments presented in chapters 3, 4 and 6 of this thesis show that competitive suppression and competitive release are pronounced when resistant parasites are at low frequency in the infection. It could well be that resistant parasites at low frequency in the infection experience more pronounced competitive suppression with increasing multiplicity of infection.

The within-host dynamics of resistant and susceptible parasites may be utilized as a resistance management tool by adjusting drug treatment regimes such that competitive suppression of drug-resistant parasites remains maintained (Wargo et al. 2007; Read and Huijben 2009; this thesis). For this, a thorough understanding of the within-host dynamics of resistant and susceptible parasites is necessary. These results suggest that clone multiplicity does not influence within-host dynamics. The generalizability of these findings needs to be studied, however, if these ecological dynamics are true for natural *P. falciparum* infections, at least one potential complexity in designing rational patient treatment regimes can be crossed off the list.

5.6 Appendix

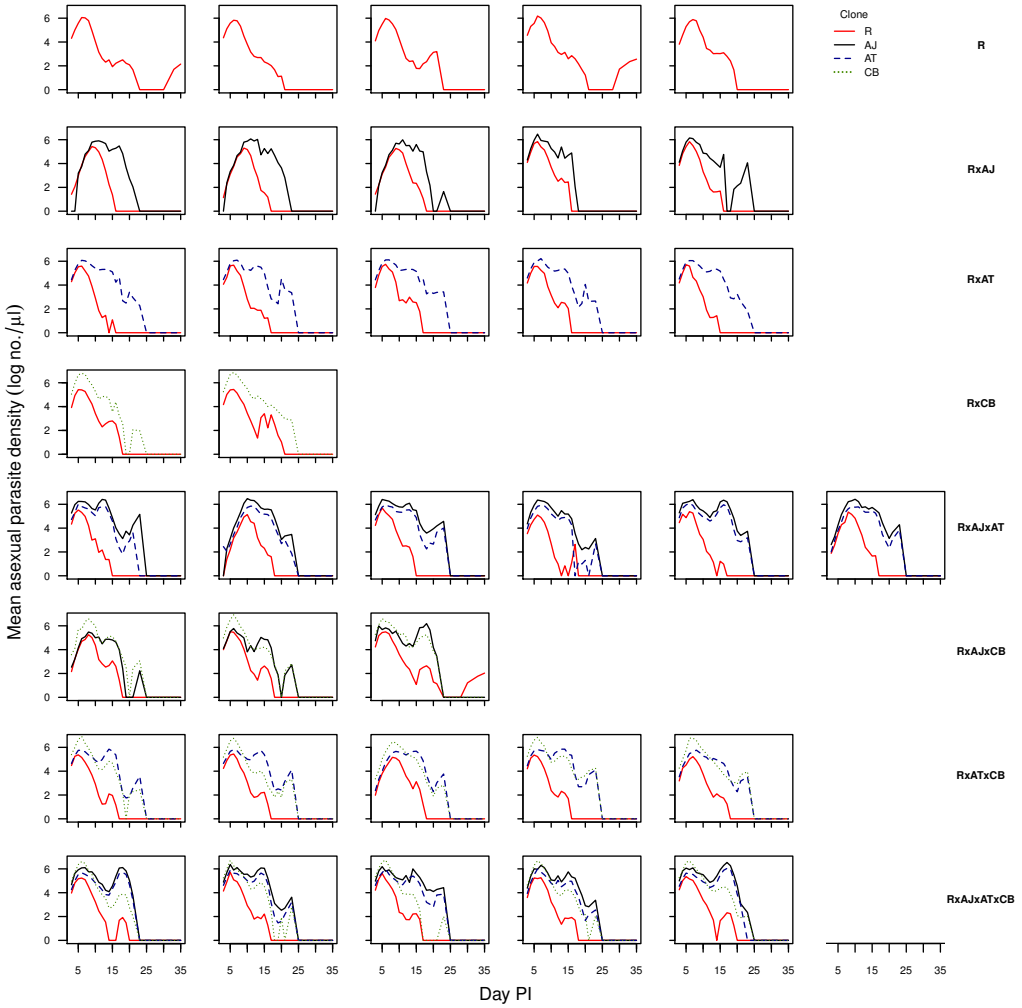

Figure A1 Parasite dynamics of individual mice in untreated infections with clone R alone (top row), or mixed clone infections of clone R + AJ (second row), clone R + AT (third row), clone R + CB (fourth row), clone R + AJ + AT (fifth row), clone R + AJ + CB (sixth row), clone R + AT + CB (seventh row) and clone R + AJ + AT + CB (bottom row). Parasite densities for clone R are shown in solid red, for clone AJ in solid black, for clone AT in dashed blue and for clone CB in dotted green. Single- and double-clone infections started out with 5 mice, three-clone infections started with 7 mice and four-clone infections with 9 mice; only surviving mice are shown (Table 5.1).

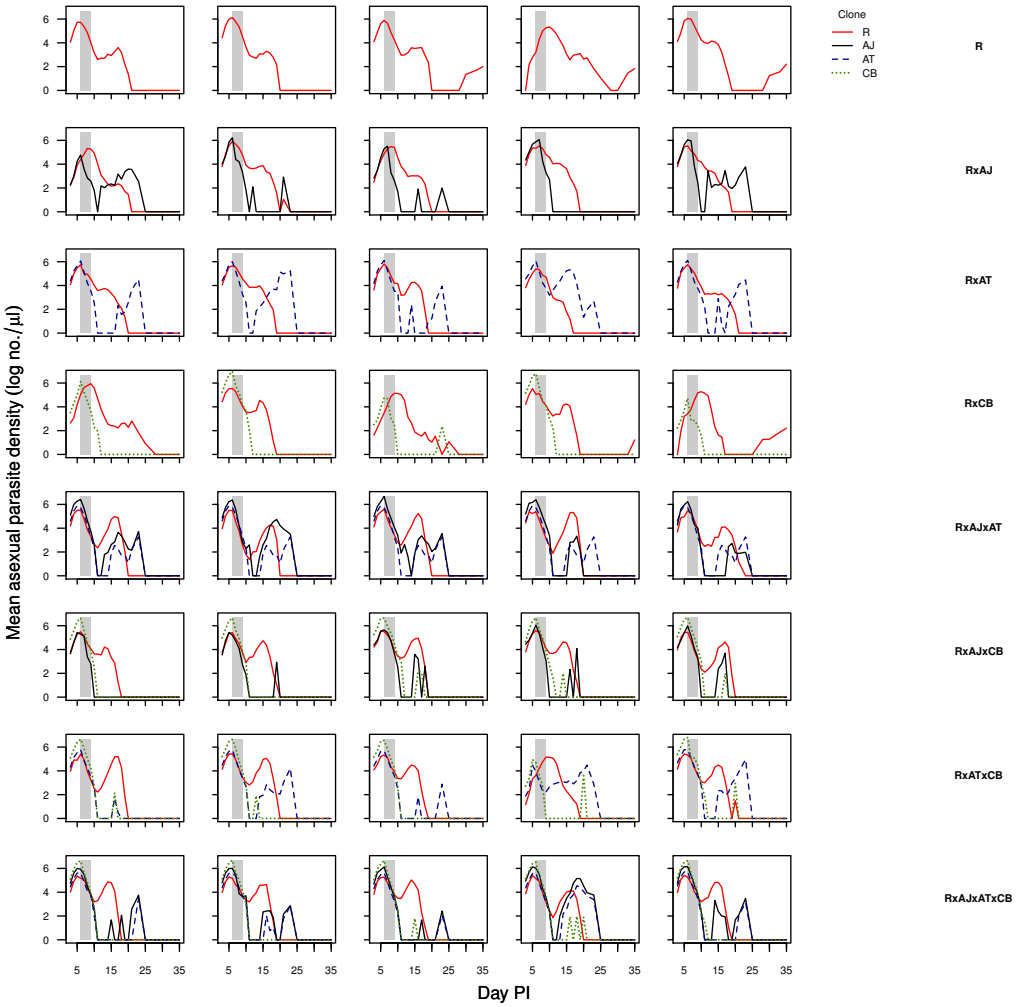

Figure A2 Parasite dynamics of individual mice in drug-treated infections with clone R alone (top row), or mixed clone infections of clone R + AJ (second row), clone R + AT (third row), clone R + CB (fourth row), clone R + AJ + AT (fifth row), clone R + AJ + CB (sixth row), clone R + AT + CB (seventh row) and clone R + AJ + AT + CB (bottom row). Parasite densities for clone R are shown in solid red, for clone AJ in solid black, for clone AT in dashed blue and for clone CB in dotted green. Drug treatment was given on days 6-9 post-infection, as indicated by the shaded area. Each treatment group consisted of 5 mice (Table 5.1).

6. The strength of within-host selection for drug resistance in malaria parasites is affected by drug treatment regime

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6.1 Abstract

Antimalarial drug treatment is aimed at improving patient health, reducing overall infectiousness, and resistance management. The current approach to achieve these objectives is radical parasitological cure, intended at killing every parasite in the infection. This approach is not obviously a resistance management strategy, as it provides the greatest possible selective advantage for any resistant parasites that are present. We hypothesize that treatment regimes which involve lower drug dosages or shorter duration of drug treatment impose weaker selection for resistant parasites while at the same time also improving patient health and reducing overall infectivity. Using a *Plasmodium chabaudi* malaria model, we found such treatment regimes are achievable. These findings raise the prospect of a different, rational, approach to developing patient treatment regimes for antimalarial resistance management.

6.2 Introduction

Drug treatment regimes of most infectious diseases have three goals: (i) improvement of patient health, (ii) reductions in transmission intensity as a public health goal and (iii) control of the emergence and spread of resistant pathogens (e.g. WHO 2006). The best treatment strategy maximizes all three of these objectives.

In the case of malaria, conventional patient treatment regimens are aimed at 'radical parasitological cure', that is drug treatment intended to kill every parasite in the infection (WHO 2006). This approach drastically reduces parasite numbers, resulting in a rapid improvement of patient health and a reduced patient infectiousness. The reduced parasite density also lowers the probability of resistance mutations appearing and minimizes the number of parasites exposed to low levels of drugs in the drug-elimination phase; in other words, reducing the selection for low level resistance (tolerance).

While this conventional patient treatment regimen may be successful at reducing the probability of new resistant mutants arising, it provides a great selective advantage to any drug-resistant parasite already present in the host, either by *de novo* mutation or by a newly derived resistant infection. The logic is as follows. When mixed infections of resistant and susceptible parasites are drug-treated, the relative fitness of resistant parasites increases due to the survival advantage they have over drug-sensitive parasites. In addition, competitive suppression of the resistant parasites by the susceptible parasites is alleviated or even fully removed following drug treatment, allowing the resistant parasites to fill up the ecological space left by susceptible parasites (Hastings 2003; Read and Huijben 2009). This competitive release has been demonstrated in *Plasmodium chabaudi* infections following both prophylactic (de Roode et al. 2004) and therapeutic (Wargo 2006; Huijben et al. *submitted*) drug treatment. Direct evidence of competitive release can not be ethically obtained for *P. falciparum* infections in humans as it requires an untreated control group, but suggestive evidence for this has been observed in *P. falciparum* infections in women receiving intermittent preventative treatment during pregnancy (IPTp; Harrington et al. 2009). Thus, from a resistance management perspective, 'radical parasitological cure' approach is a two-edged sword: it reduces the probability of resistant mutants arising, but at the same time maximally selects for the resistant parasites already present (Hastings 2003; Read and Huijben 2009). In the case of malaria, *de novo* resistance mutational events are extremely rare; for most drugs, global resistance is the result of a few resistant strains that have swept across most parts of the malaria endemic world (reviewed in Plowe 2009). Therefore, conventional patient treatment regimes may be obstructing the third objective of patient treatment, namely resistance management.

Since we know competitive release provides a great advantage to resistant parasites, and that shorter treatment regimens (Wargo et al. 2007) and reduced drug dosages (Huijben et al. *submitted*) reduce the extent of competitive release, we wanted to test the hypothesis that treatment regimens other than radical parasitological cure may be better at simultaneously maximizing the three treatment goals when resistance is already present in the population. Here our aim is not, of course, to find the best treatment regimen for *falciparum* malaria infections, but rather to test a proof of principle: compared to radical parasitological cure, are there treatment regimens that better maximize simultaneously host health, reductions in infectiousness and resistance management? We found that there are.

6.3 Material and methods

A mathematical model was utilized in the decision process for choosing drug regimes to test experimentally. For details on this model, see appendix to this chapter.

6.3.1 Parasites and hosts

Two genetically distinct clonal lineages were used in the experiments, drug-sensitive AJ_{5p} (hereafter referred to as clone S) and drug-resistant clone $AS_{6p(pyr1A)}$ (hereafter referred to as clone R), both clones were isolated from thicket rats and subsequently cloned (Beale et al. 1978). Clone R was made resistant by exposing it to a high dose of pyrimethamine in a single passage (Walliker et al. 1975). Hosts were nine week old female C57Bl/6 laboratory mice (Charles River Laboratories). All mice were kept on a 12:12 L:D cycle, fed Laboratory Rodent Diet 5001 (LabDiet, PMI Nutrition International) and received 0.05% PABA-supplemented drinking water to enhance parasite growth (Jacobs 1964).

6.3.2 Experimental design, infections and drug treatment

Mice were randomly assigned to one of eight treatment groups. Each group consisted of eight mice, randomly allocated to two cages with four mice in each cage. All mice were inoculated at day zero with a mixed infection of both clone R and clone S, so as to establish an infection with 10^6 parasites of clone S and ~10 parasites of clone R,

representing a situation where resistance is rare in the population. On day 6 post-infection (PI), at the onset of disease symptoms, mice received:

i) no drug treatment,

ii) 8 mg/kg pyrimethamine ("high dose"), given 5 days in a row ("conventional treatment"),

or one of six alternative treatment regimes:

iii) 4 mg/kg pyrimethamine ("low dose"), given only once,

iv) 4 mg/kg pyrimethamine, given every 7 days,

v) 4 mg/kg pyrimethamine, given every 4 days,

vi) 8 mg/kg pyrimethamine, given only once,

vii) 8 mg/kg pyrimethamine, given every 7 days, or

viii) 8 mg/kg pyrimethamine, given every 4 days.

Pyrimethamine was dissolved in dimethyl sulfoxide (DMSO) and inoculated intra peritoneal with 50 µl of the solution. The untreated control group did not receive any treatment during the entire infection to mimic the situation of untreated patients.

6.3.3 Monitoring of infections

Weight, red blood cell density, asexual parasite density and gametocyte density of both clones were measured daily (day 3-22 PI) and three times a week thereafter (day 24-49 PI). Weight was determined to the nearest 0.1 gram. Red blood cell density was measured using flow-cytometry (Beckman Coulter), for which two µl of tail snip blood was diluted in 80 ml isotonic saline. A subsequent 5 µl and 10 µl of tail snip blood was taken for respectively asexual parasite and gametocyte quantification.

DNA was extracted from 5 µl of blood on the ABI Prism® 6100 Nucleic Acid PrepStation according to manufacturer's instructions. RNA was extracted from 10 µl of blood using the 'RNA Blood-DNA' method on the ABI Prism® 6100 Nucleic Acid PrepStation. RNA was subsequently converted to single stranded cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Wargo et al. 2006). Both DNA and cDNA were stored at -80°C until quantification.

To measure total parasite density (asexual parasites and gametocytes combined), quantitative PCR was performed on DNA using clone-specific assays. To measure gametocyte density, the same clone-specific quantitative PCR assays were performed

on cDNA (Drew and Reece 2007). Asexual parasite density was estimated by subtracting the gametocyte density from the total parasite density. Each reaction, with a final volume of 25 µl consisted of 7 µl DNA or cDNA, 900 nM forward and reverse clone-specific primers, 250 nM TaqMan® MGB probe (Applied Biosystems) and 1x PerfeCTa™ qPCR FastMix™ (Quanta Biosciences). Primer and probe sequences are those used by Drew and Reece (2007). All reactions were run on the ABI Prism® 7500 Fast System, using the assay: 95°C for 2 minutes, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Quantification was based on serial dilutions of DNA and cDNA standards of known parasite and gametocyte density respectively, determined beforehand by microscopy.

6.3.4 Statistical analysis

Analyses were performed in R 2.9.0 (R Development Core Team 2009). The geometric mean asexual parasite densities of clone R and S separately and combined were calculated for the period after the initiation of drug treatment (day 7-49 PI). Since asexual parasite densities range over 6 orders of magnitude during the course of the infection, mean asexual densities were summarized as the geometric mean. As a measure of transmission potential, the predicted infectiousness was calculated for both clones separately and combined after the start of treatment (day 7-49 PI) using gametocyte densities in the density-infectivity function q1 as described in the general introduction of this thesis. The rate of decline of asexual S parasites after the first day of treatment was calculated as the difference in the log-transformed densities of day 6 and 7. Additionally, gametocyte density following 8 mg/kg, 4 mg/kg treatment and no drug treatment was compared, for which the proportion of the gametocyte density on day 6 (prior to treatment) compared to the gametocyte density on day 7 (first day post-treatment) was estimated and subsequently square root transformed to meet normality assumptions. The change in mouse body mass, as a percentage from the baseline value of the mean body mass of day -1 and day 3 for each mouse, was calculated through time. As measurements of host health, the mean body mass change and mean red blood cell (RBC) density post-treatment (day 7-49 PI) were calculated. Furthermore, the maximum loss in body mass and minimum RBC density during the acute infection (day 3 – 11 PI) and during the parasite relapse (day 12-49 PI) were taken.

Analysis of variance (ANOVA) was used to test for a difference between drug-treated and untreated infections. A subsequent ANOVA was applied to test for an effect of drug treatment regime among treated infections. Post-hoc Tukey Honestly Significant Differences (HSD) tests were carried out for pair-wise comparisons of treatment regimes, in which case the adjusted p-values are reported. Lastly, a fully cross-factored ANOVA was done on the alternative treatments with drug dose (*low*: 4 mg/kg, or *high*: 8 mg/kg) and treatment frequency (*once*/ *every 7 days*/ *every 4 days*). To ensure treatment groups did not differ prior to drug treatment, day 6 values on asexual parasite density, gametocyte density, proportional change in body mass and RBC density were evaluated for statistical differences. Significant differences between treatment groups were found in gametocyte densities of clone S and R+S combined and in RBC densities, therefore, day 6 values of these variables were included in their respective models as a covariate.

A total of 15 mice failed to become infected with clone R (determined by the absence of detection by qPCR during the entire course of infection), presumably as a result of stochastic loss due to the low inoculum size. These mice had therefore to be removed from the analysis. Another mouse received a substantially lower inoculum of clone S than intended (treatment group 4 mg/kg every 4 days), as judged from much lower parasite densities the first 6 days post-infection than is expected for a 10^6 parasite dose (Chapter 3), and was therefore also removed from the analysis (Table 6.1).

Table 6.1 Treatment regimes used in the experiment with number of mice at the start of each treatment. The number in brackets indicates the number of mice used in the analysis (see material and methods).

Pyrimethamine dose	1 day only	every 4 days	every 7 days	5 days in a row	none
4 mg/kg	8 (7)	8 (6)	8 (5)		
8 mg/kg	8 (7)	8 (5)	8 (6)	8 (5)	
untreated					8 (7)

6.4 Results

6.4.1 Asexual parasite dynamics

In untreated infections, resistant parasites, which were inoculated at five orders of magnitude lower than the susceptible parasites, barely reached densities above

detection (Figure 6.1a). In the absence of competition, this parasite dose results in similar levels of peak parasite density as a parasite dose five orders of magnitude larger (Chapter 3). Drug treatment of these mixed infections resulted in a release of resistant parasites, though the level of release varied greatly with drug treatment regime (Figure 6.1b-h).

Drug treatment significantly reduced susceptible parasite densities ($F_{1,46}$=7.6, p=0.009), the extent of which varied among treatment regimes (Figure 6.1, 6.2a; $F_{6,34}$=5.8, p<0.001). Conventional treatment led to the greatest suppression of susceptible parasites, with a significantly lower mean asexual parasite density than all other treatments, except for the 8 mg/kg dose every 7 days treatment (Figure 6.3a). This enhanced suppression of susceptible parasites following conventional treatment resulted in a greater release of resistant parasites. A similar or higher resistant parasite density was produced in this treatment group than in other treatment groups (Figure 6.1, 6.2b, 6.3b; $F_{6,34}$=5.0, p<0.001). Analysis of the proposed alternative treatment regimes showed that drug dose had a significant effect on the release of resistant parasites (*dose*: $F_{1,32}$=8.0, p=0.008), while the frequency of treatment did not (*frequency*: $F_{2,32}$=2.3, p=0.12).

Resistant parasites typically showed only a single parasite peak following competitive release, while the susceptible parasites rebounded after drug pressure wore off and subsequently had a third parasite peak after day 20 PI (Figure 6.1). The rate of decline of susceptible parasites following drug treatment was similar following a low dose (4 mg/kg) and high dose (8 mg/kg) treatment (Tukey HSD, p_{adj}=0.27), in both cases much more rapidly than in untreated control mice (Figure 6.2a, $F_{2,45}$=18, p<0.001) . However, the drug-induced decline persisted for longer in high dose treatments. Drug treatment with a high dose for 5 days in a row (conventional treatment) resulted in a prolonged decline, which resulted in the susceptible parasites in all mice being below detection level by day 11. However, also these susceptible parasites relapsed after drug pressure wore off, coinciding with the relapse of resistant parasites.

Taken together, drug-treated infections had a slightly lower total asexual parasite density than untreated infections (Figure 6.3c; $F_{1,46}$=5.1, p=0.029). However, among the different treatment regimes, no differences were observed in total parasite density

($F_{6,34}$=0.7, p=0.69). These similar parasite densities among treatment groups are the result of high numbers of susceptible parasites and low numbers of resistant parasites in the low dose treatment groups and vice versa in the high dose treatment groups.

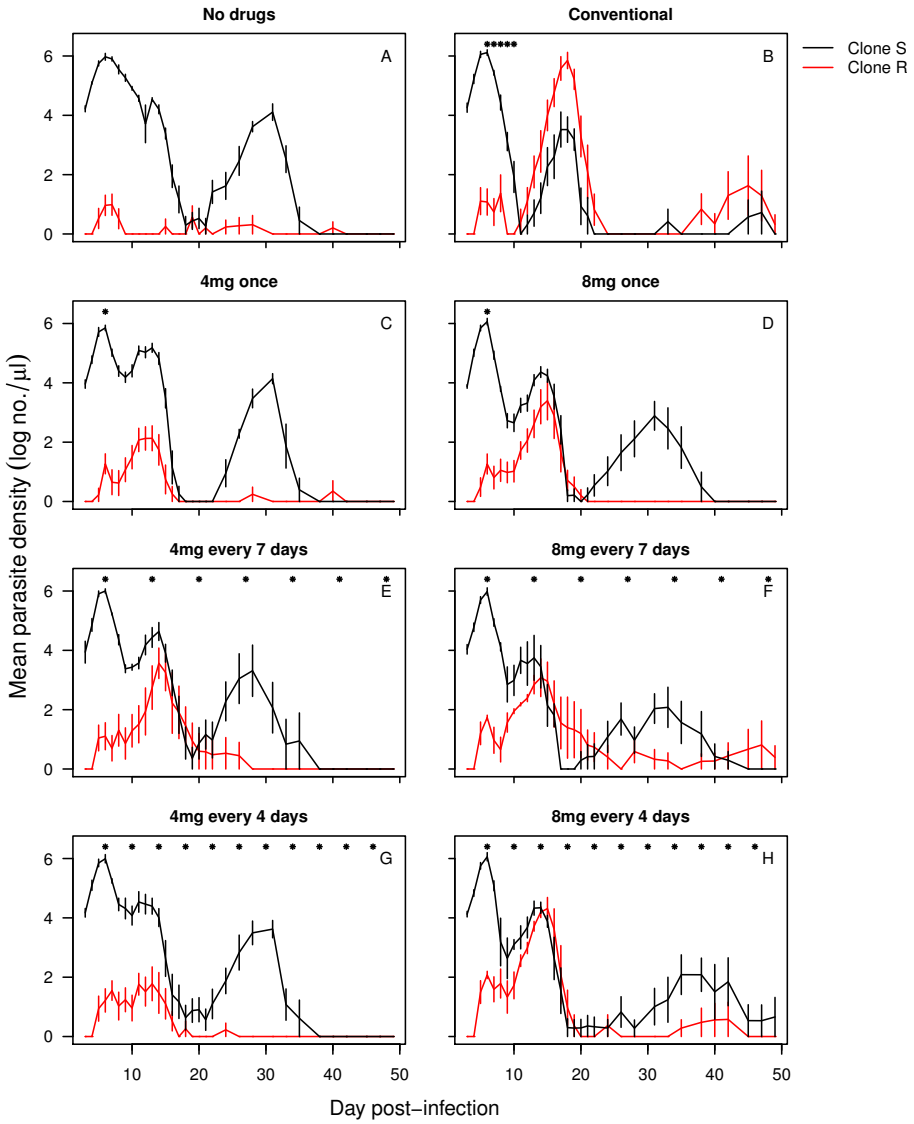

Figure 6.1 Asexual parasite dynamics of drug-resistant clone R (red line) and drug-sensitive clone S (black line). Infections were either left untreated (a), treated with 8 mg/kg pyrimethamine for 5 days in a row ("Conventional") (b), or received one of the alternative treatments: 4 mg/kg only once (c), 8 mg/kg only once (d), 4 mg/kg every 7 days (e), 8 mg/kg every 7 days (f), 4 mg/kg every 4 days (g) or 8 mg/kg every 4 days (h). Days of drug treatment are indicated by asterisks. Data are geometric means (± standard error) of 5-7 mice (Table 6.1).

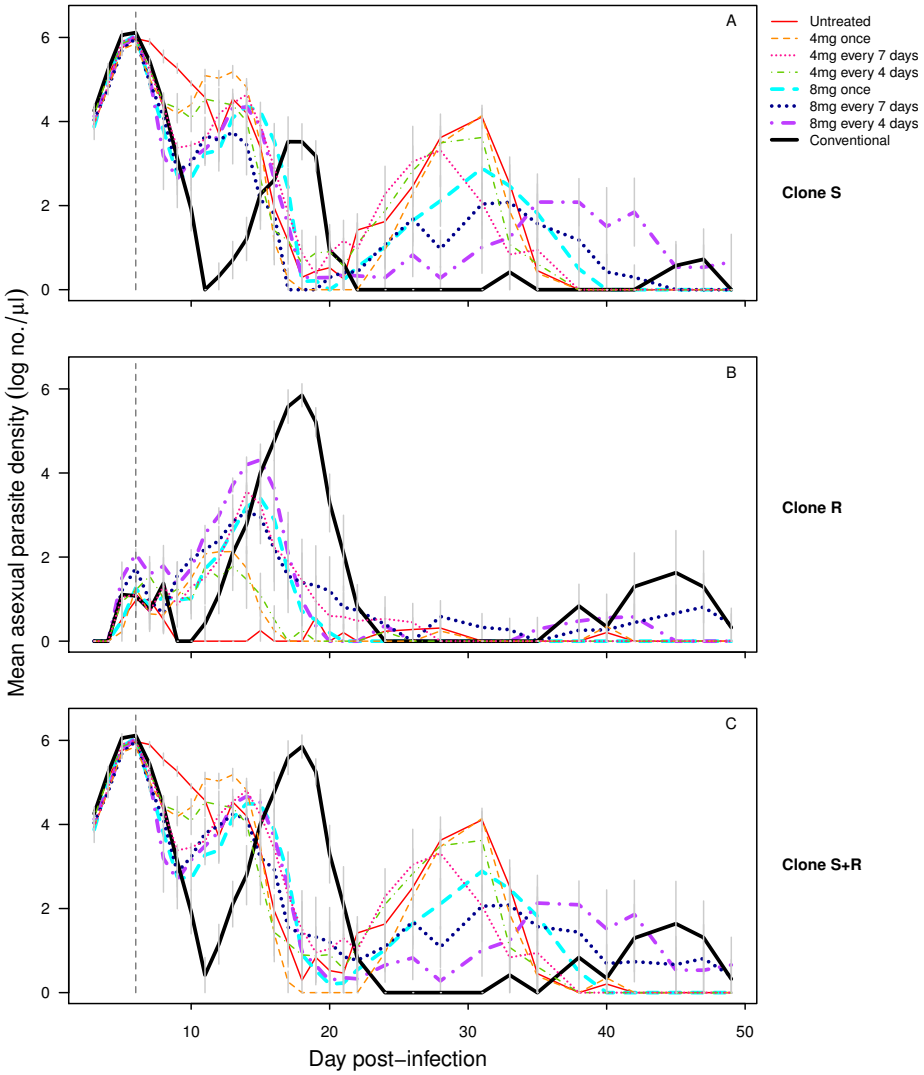

Figure 6.2 Asexual parasite dynamics of clone S (upper panel), clone R (middle panel) and clone R+S combined (bottom panel) of untreated infections (solid thin red line), infections treated with a low dose of drugs (4 mg/kg) given only once (dashed thin orange line), every 7 days (dotted thin pink line), or every 4 days (dotdashed thin green line) and infections treated with a high dose of drugs (8 mg/kg) given only once (dashed thick cyan line), every 7 days (dotted thick blue line), every 4 days (dotdashed thick purple line) or for 5 days in a row only ("Conventional treatment", solid thick black line). Drug treatment was in all cases initiated on day 6 PI, as indicated by the dashed grey line. Data are geometric means (± standard error) of 5-7 mice (Table 6.1).

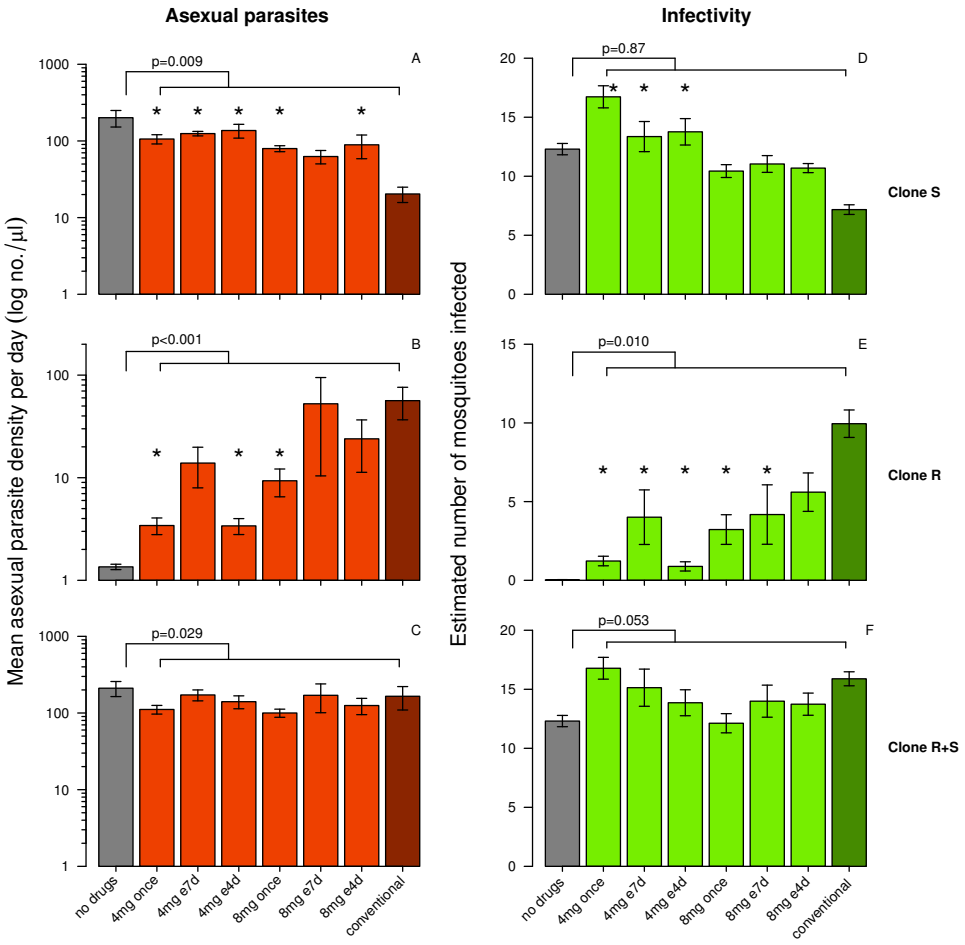

Figure 6.3 Geometric mean (± standard error) asexual parasite density per day after start of treatment (left panels) and arithmetic mean (± standard error) predicted number of infected mosquitoes from a total of n=100 after start of treatment (right panels) of clone R (upper panels), clone S (middle panels) and clone R+S combined (bottom panels) for each treatment group. Treatment groups (x-axis) were no treatment (left hand bar in gray), 8 mg/kg for 5 days in a row ("conventional treatment", right hand bar in dark red and dark green), or one of the alternative treatments: 4mg/kg given only once ("4mg once"), 4 mg/kg given every 7 days ("4mg e7d"), 4 mg/kg given every 4 days ("4mg e4d"), 8 mg/kg given only once ("8mg once), 8 mg/kg given every 7 days ("8mg e7d") and 8 mg/kg given every 4 days ("8mg e4d"). Data are means of 5-7 mice (Table 6.1). The p-values indicate the level of significance of the difference between untreated and all drug-treated groups together. The asterisks point out the alternative treatment groups which differ from conventional treatment (based on post-hoc Tukey HSD tests with $p_{adj} < 0.05$). Note the y-axis varies between plots.

6.4.2 Transmission potential

Clone R did not produce gametocytes in untreated infections, while clone S typically produced three distinct gametocyte peaks throughout the infection: the first coinciding with peak asexual parasite densities, a second around day 12 and a third after day 20 post-infection (Figure 6.4a, 6.5a). As a result of competitive release of resistant parasites, clone R produced a single gametocyte peak following drug treatment, coinciding with the asexual parasite relapse and the level of release of gametocytes dependent on the drug regime (Figure 6.4b-h, 6.5b).

Importantly, drug treatment with a high dose resulted in increased gametocyte density the day following drug treatment, whereas untreated infections showed a drop in gametocyte density ($p_{adj}<0.001$). A difference between untreated and low dose treatment was also found ($p_{adj}=0.005$), with the latter having similar gametocyte densities before and after treatment (inset graph figure 6.5a). Also, the single low dose treatment resulted in a greatly increased second gametocyte peak of susceptible parasites compared to all other treatment regimes (Figure 6.5a).

Large differences between different treatment regimes were thus found in the predicted transmission of clone S (Figure 6.3d), with all low dose treatments resulting in higher predicted transmission than conventional treatment. Among the alternative treatments, drug dose had the greatest impact on transmission of susceptible parasites (*dose*: $F_{1,29}=33$, $p<0.001$), whereas frequency of treatment did not make a difference (*frequency*: $F_{2,29}=1.7$, $p=0.20$).

Drug treatment greatly increased the transmission potential of drug-resistant parasites ($F_{1,46}=7.3$, $p=0.010$). Conventional treatment provided the greatest predicted infectivity for resistant parasites than all other treatments, with the exception of 8 mg/kg given every four days (Figure 6.3e). Among the different alternative treatment regimes, a higher drug dose was the biggest determinant for increased transmission potential for resistant parasites ($F_{1,32}=7.0$, $p=0.012$). A higher frequency of treatment did not increase the predicted infectivity of clone R ($F_{2,32}=1.1$, $p=0.33$).

Altogether, drug treatment tended to increase the overall infectiousness of the mixed infections ($F_{1,45}=4.0$, $p=0.053$). No differences were found in the overall infectiousness

between conventional treatment and the alternative treatments (Figure 6.3f), which, as in the asexual densities, is the result of the balance between high transmission potential of susceptible parasites and low transmission of resistant parasites or vice versa, depending on treatment regime.

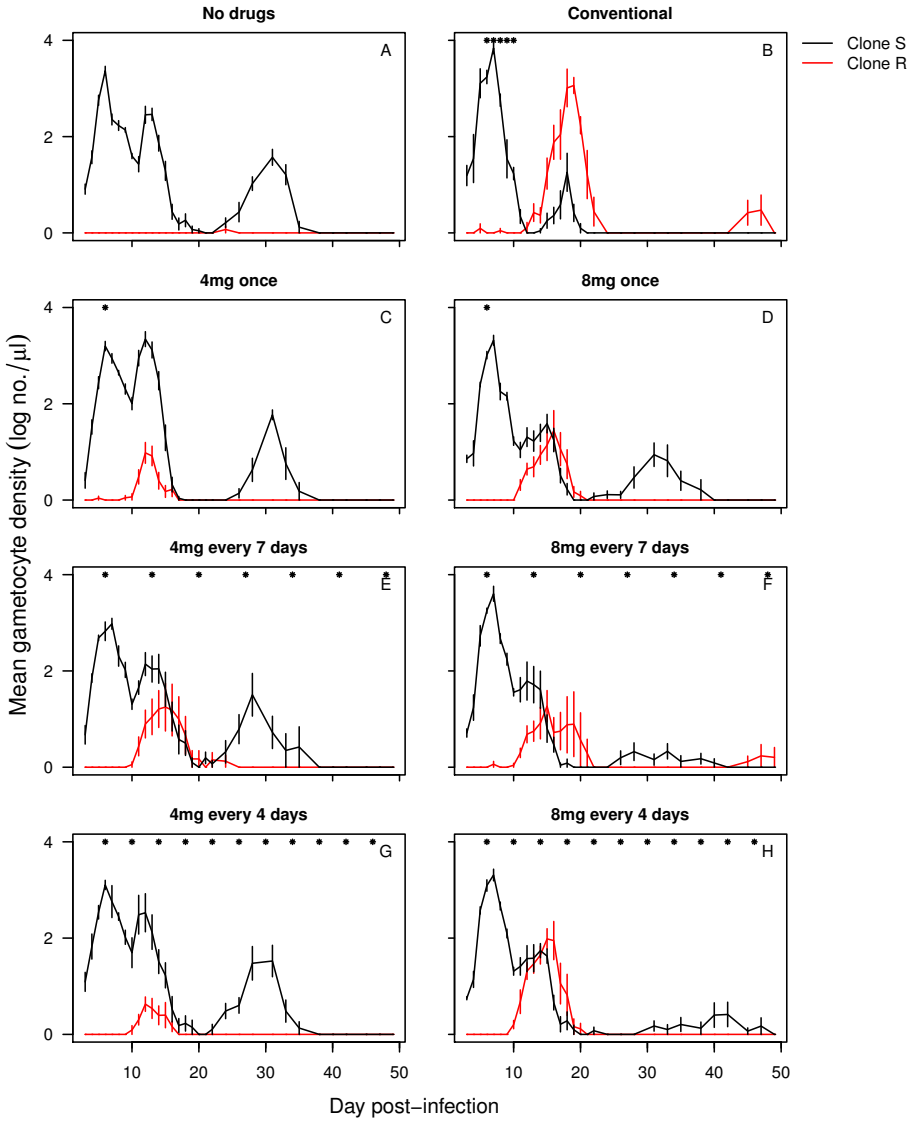

Figure 6.4 Gametocyte dynamics of drug-resistant clone R (red line) and drug-sensitive clone S (black line). Infections were either left untreated (a), treated with 8 mg/kg pyrimethamine for 5 days in a row ("Conventional") (b), or received one of the alternative treatments: 4 mg/kg only once (c), 8 mg/kg only once (d), 4 mg/kg every 7 days (e), 8 mg/kg every 7 days (f), 4 mg/kg every 4 days (g) or 8 mg/kg every 4 days (h). Days of drug treatment are indicated by asterisks. Data are geometric means (± standard error) of 5-7 mice (Table 6.1).

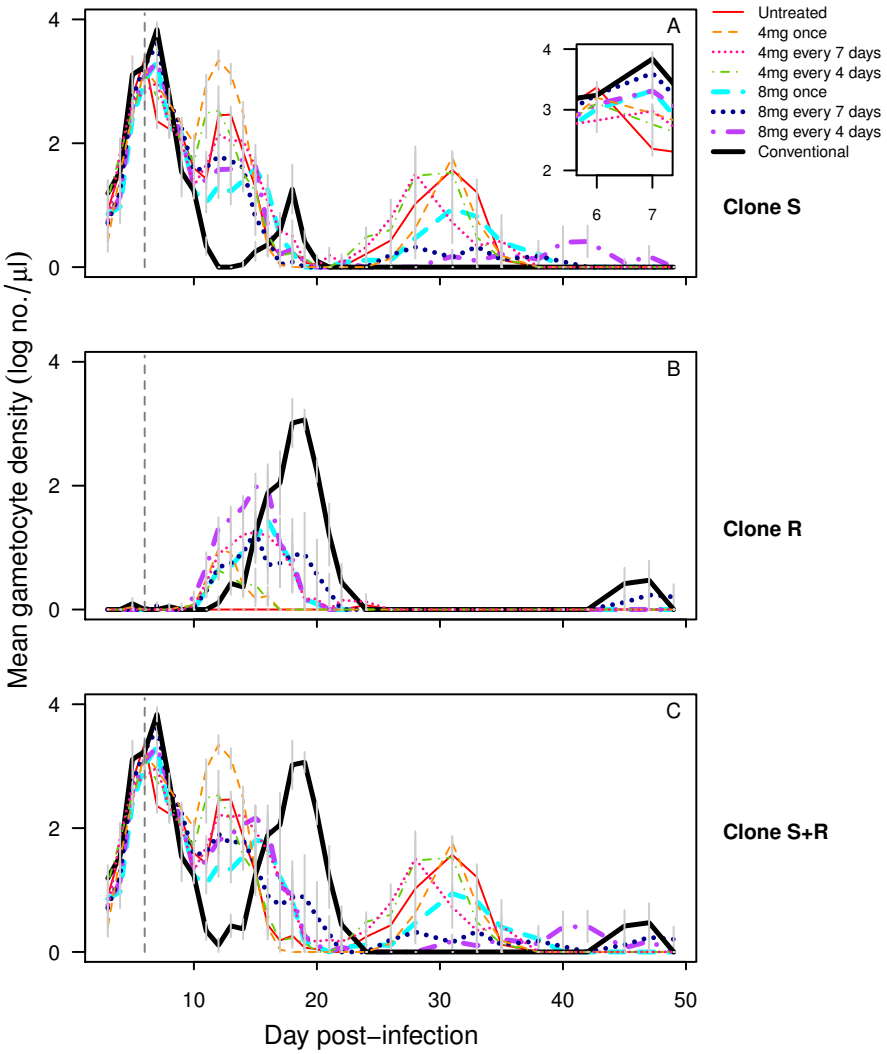

Figure 6.5 Gametocyte dynamics of clone S (upper panel), clone R (middle panel) and clones R+S combined (bottom panel) of untreated infections (solid thin red line), infections treated with a low dose of drugs (4 mg/kg) given only once (dashed thin orange line), every 7 days (dotted thin pink line), or every 4 days (dotdashed thin green line) and infections treated with a high dose of drugs (8 mg/kg) given only once (dashed thick cyan line), every 7 days (dotted thick blue line), every 4 days (dotdashed thick purple line) or for 5 days in a row only ("Conventional treatment", solid thick black line). The inset graph in the upper panel is an enlargement of day 6 and 7 PI. Drug treatment was in all cases initiated on day 6 PI, as indicated by the dashed grey line. Data are geometric means (± standard error) of 5-7 mice (Table 6.1).

6.4.3 Host health

Drug treatment led to earlier recovery from anaemia and body mass loss during the acute phase of the infection compared to untreated infections, which resulted in less weight loss and anaemia in drug-treated than in untreated infections (Figure 6.6, 6.7ab, minimum red blood cell density: $F_{1,45}$=66, p<0.001; maximum proportional body mass loss: $F_{1,46}$=4.2, p=0.045;). However, drug-induced parasite relapses also affected host-health, with a relapse in anaemia coinciding with parasite relapse (Figure 6.6b, 6.7c). Parasite relapses did not affect mouse body mass (Figure 6.6a, 6.7d). The anaemia relapses were most severe following conventional treatment, though no significant differences were observed with the alternative treatments, with the exception of high dose treatment given every 4 days (Figure 6.7C). Importantly, frequency of drug treatment had a significant impact on the severity of the anaemic relapse; more frequent treatment resulted in reduced red blood cell loss (*frequency*: $F_{2,31}$=3.7, p=0.037), while treatment dose did not have an impact on the severity of the anaemic relapse (*dose*: $F_{1,31}$=0.1, p=0.78). Overall, no differences were observed between conventional treatment and alternative treatments on the average red blood cell density and change in body mass after the start of treatment regimes.

Of note is that mice which were treated less frequently throughout the infection (no treatment, treatment given only once and conventional treatment given 5 days in a row early in the infection), increased in body mass at a slightly higher rate than mice that were treated more frequently (Figure 6.6a). This observation is likely a result of treatment stress. We deliberately chose not to include control mice for each treatment or impose treatment stress on all mice for each treatment to reduce the number of mice and overall stress. This stress response was not observed in red blood cell densities.

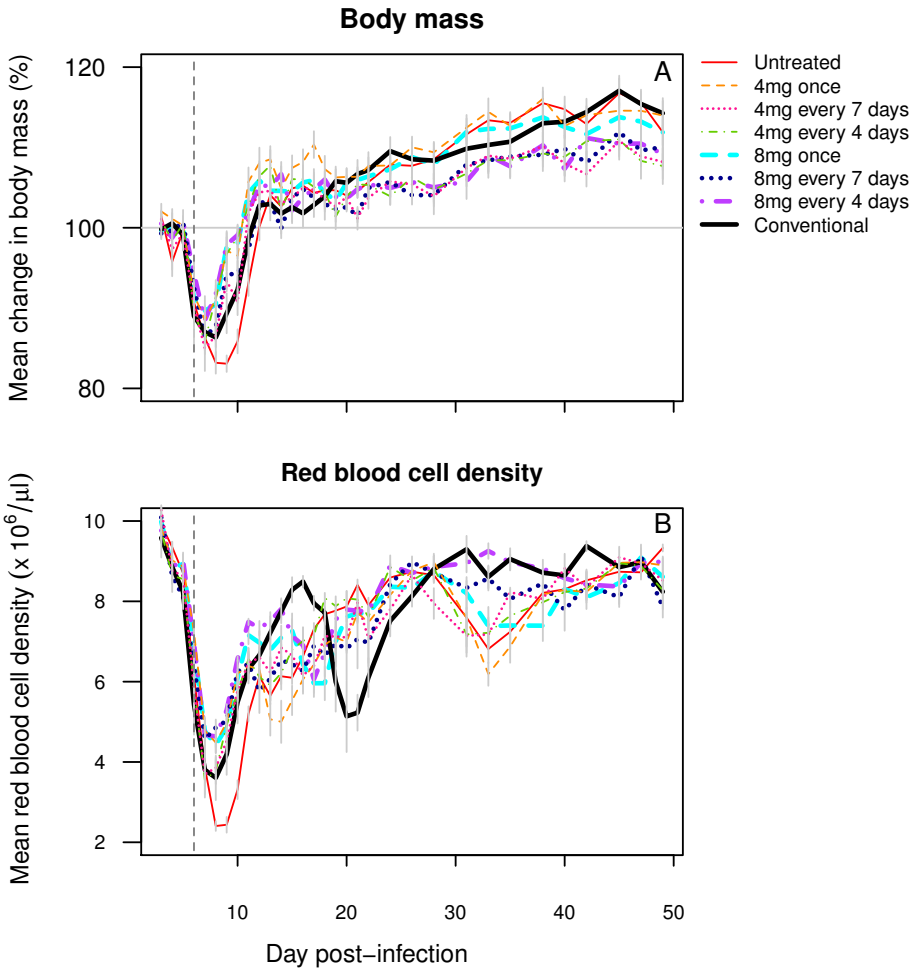

Figure 6.6 Mean change in body mass (upper panel) and red blood cell densities (lower panel) through time of untreated infections (solid thin red line), infections treated with a low dose of drugs (4 mg/kg) given only once (dashed thin orange line), every 7 days (dotted thin pink line), or every 4 days (dotdashed thin green line) and infections treated with a high dose of drugs (8 mg/kg) given only once (dashed thick cyan line), every 7 days (dotted thick blue line), every 4 days (dotdashed thick purple line) or for 5 days in a row only ("Conventional treatment", solid thick black line). Drug treatment was in all cases initiated on day 6 PI, as indicated by the dashed grey line. Data are means (± standard error) of 5-7 mice (Table 6.1).

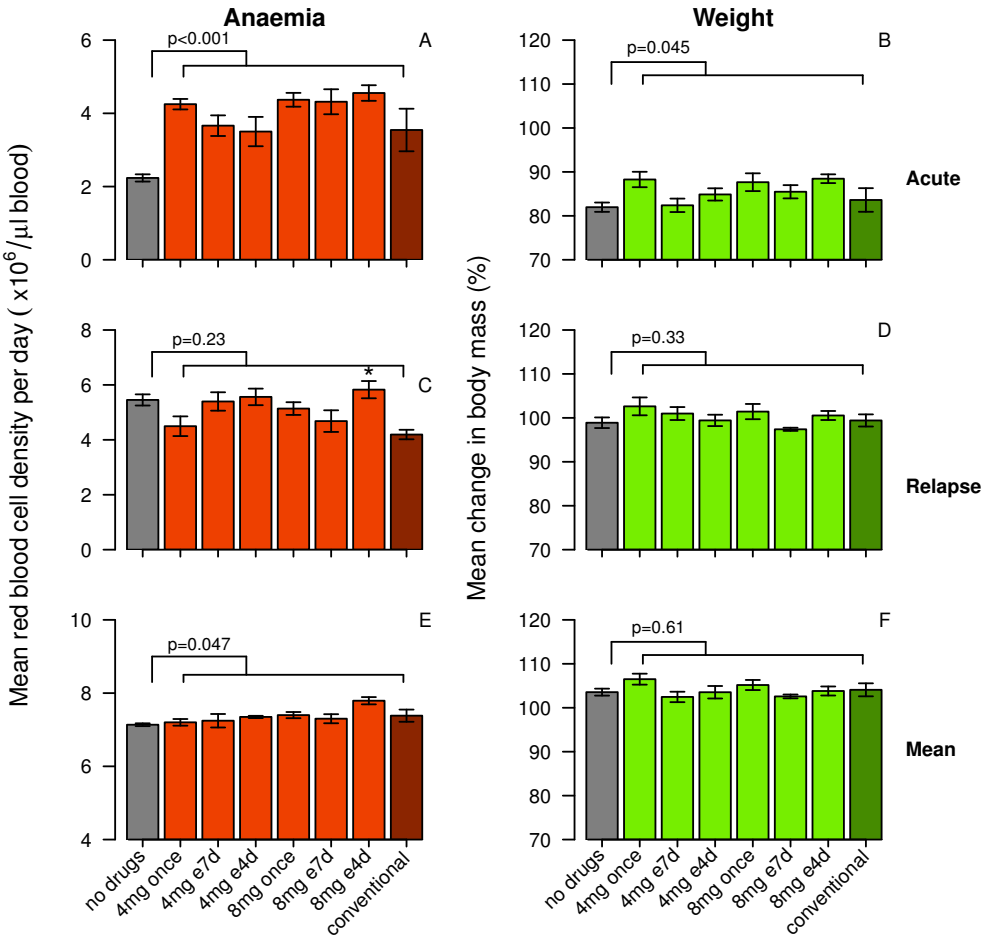

Figure 6.7 Red blood cell density (left panels) and change in body mass (right panels) per treatment group. The upper panels show the mean minima reached during the acute phase (day 3 to 11), the middle panels show the mean minima reached during relapse (day 12 to 25), and the bottom panels show the mean values over the course of infection after the start of treatment (day 7-49 PI). Treatment groups were no treatment, 8 mg/kg for 5 days in a row ("conventional treatment"), or one of the alternative treatments: 4mg/kg given only once ("4mg once"), 4 mg/kg given every 7 days ("4mg e7d"), 4 mg/kg given every 4 days ("4mg e4d"), 8 mg/kg given only once ("8mg once), 8 mg/kg given every 7 days ("8mg e7d") and 8 mg/kg given every 4 days ("8mg e4d"). Data are arithmetic means of 5-7 mice (Table 6.1). The p-values indicate the level of significance of the difference between untreated and all drug-treated groups together. The asterisks point out the alternative treatment groups which differ from conventional treatment. Note the y-axis varies between plots.

6.5 Discussion

In this experiment, we tested the hypothesis that drug treatment regimes other than the conventional approach of radical parasitological cure could perform better at simultaneously maximizing all three treatment goals: (i) improvement of patient health, (ii) reduction in infectiousness, and (iii) resistance management. The data presented here provide proof of principle that there are other treatment strategies which do this. Reduced drug dosages resulted in less competitive release of resistant parasites, which furthermore resulted in a lower predicted transmission potential for resistant parasites (resistance management). High dose treatment reduced transmission potential of susceptible parasites but increased the transmission potential of resistant parasites. The reverse was observed in low dose treatment. Therefore, the overall predicted infectivity of both clones combined was comparable across the diverse treatment regimes. All treatment regimes reduced host morbidity as well as conventional treatment, though conventional treatment caused pronounced anaemia during relapse. Together, these data show that, in principle, there are treatment regimes other than radical parasite cure, which perform equally well or even better at improving host health and reducing transmission, while more effectively reducing the predicted infectivity of the resistant parasites.

Thus, our data suggest that current treatment regimes may not be the best approach to treat patients with malaria if these results generalize to natural human infections. In our experiment, reduced treatment was enough because host immune system rapidly controlled the infection. This might take longer in *P. falciparum* infections. Additionally, the 'best' treatment likely depends on a variety of factors. The infections in this study were seeded with a low abundance of drug-resistant parasites. Hence, resistance was already present. If resistance had not been present in the infection, conventional treatment would have likely been the best treatment at controlling parasite populations and reducing overall infectivity (Figures 3ad). The best rational treatment regime may thus depend on the presence of resistant mutants, whereby for instance a change in treatment practice from conventional treatment to a reduced treatment regime is advised once resistant parasites have been observed in a region. The dramatic effect of continuing drug treatment in an area with high level drug resistance was demonstrated using the effect of IPTp. These intermittent treatments work very well in areas with low levels of resistance (ter Kuile et al. 2007), however,

when resistance is wide spread, IPTp seems to exacerbate malaria infections in pregnant women (Harrington et al. 2009).

Reducing drug dose or shortening the length of treatment to avoid the elimination of all susceptible parasites from an infection and therefore reducing the selection for resistance contradicts with medical orthodoxy. However, this orthodoxy is being challenged in different areas of research. In cancer treatment for instance, a recent study has shown that low but frequent drug dosages are the best way of controlling resistant cells from growing. This type of treatment resulted in 100% survival of mice infected with a human ovarian cancer, whereas all untreated and conventionally (high-dose) treated mice died (Gatenby 2009; Gatenby et al. 2009). An *in vitro* study using the *Staphylococcus aureus* bacteria showed that a short course of antibacterial drugs performed better at controlling the resistant bacterial population than the conventional longer course (Drusano et al. 2009).

Despite these recent studies questioning orthodox belief that the causal agent should be hit as hard as possible, the translation of the findings presented in this experiment to refinement of current treatment protocols for malaria is many steps away. The mouse malaria model system is, like any model of a human disease, an approximation of the reality and may not capture certain relevant factors. The findings in this study should therefore be interpreted with caution and merely be seen as a proof of principle, which, in due course, may lead to clinical trials. Before then, more empirical testing is necessary to establish the generality of these findings among a variety of parasite strains in both animal and *in vitro P. falciparum* models.

Of note is that increased gametocyte densities following the first day of drug treatment were observed for susceptible parasites, with the highest densities following high dose treatment (Figure 6.5a). Such effect was also observed in the experiment described in chapter 2 of this thesis. This observation could be the result of so-called drug-induced gametocytogenesis as a response to conditions unfavorable for asexual replication. However, increased gametocyte densities immediately following treatment are not necessarily a result of gametocytogenesis, since gametocyte maturation in *P. chabaudi* is thought to take more than 24 hours (Carter and Graves 1988). An alternative explanation could be reduced gametocyte clearance rates following drug treatment.

Increased gametocyte densities following drug treatment have been observed before with pyrimethamine treatment in *P. chabaudi in vivo* (Buckling et al. 1999a), in *P. falciparum* following chloroquine treatment *in vitro* (Buckling et al. 1999b) and has been suggested multiple times in falciparum malaria in the field following sulphadoxine-pyrimethamine drug treatment (Puta and Manyando 1997; Robert et al. 2000; Osorio et al. 2002; Sowunmi and Fateye 2003; Talman et al. 2004; Ali et al. 2006; Sowunmi et al. 2006), although these field data are difficult to interpret due to the absence of untreated controls. This facultative response, which appears to be dose dependent, can be important for the spread of drug resistance, since it reduces the relative fitness of drug-resistant parasites. The specificity of these findings to pyrimethamine should be further studied.

An unexpected observation in this study was the low impact of drug treatment on susceptible parasites later on in the infection (Figure 6.1: susceptible parasite densities were increasing despite administered drug treatments). There are several possible explanations for this observation. First, the parasites from the susceptible strain could (partly) consist of drug-resistant mutants. Selection for resistance against pyrimethamine is very likely to occur after only a single exposure to this drug (Walliker et al. 1975). However, *de novo* resistant mutants are unlikely to be present at high densities early in the infection and are thus unlikely the cause for reduced sensitivity to high dose drug treatment as early as for instance 10 days post-infection (Figure 6.1h). Another explanation could be an enhanced drug detoxification by the mouse after multiple exposures, though we are unaware of any evidence for this mechanism. A specific characteristic of pyrimethamine is that it only kills replicating parasites (Foote and Cowman 1994). Therefore, a further explanation could be a persistence of temporarily growth-arrested intra-erythrocytic parasites (White 2008), or reduced growth rate or burst size later on in the infection resulting in reduced susceptibility. Furthermore, the synchronicity of *P. chabaudi* parasites is known to break down somewhat as the infection progresses (Mackinnon et al. 2002b). Therefore, a shift in the time of day of parasite replication could result in lower drug concentrations at the time of replication, since the half-life of pyrimethamine is estimated to be only 4.5 hours (Coleman et al. 1986). Finally, there could be a density-dependent drug-efficacy effect, since the drug-efficacy was high at peak parasite density and evidently less effective at lower densities.

Clearly, this is an issue that needs to be resolved. Further studies could demonstrate drug efficacy at different time points in an infection and the effect of multiple exposures on parasite reduction. Additional studies using different classes of drugs could show whether this is a pyrimethamine-specific effect. Understanding the mechanism behind reduced drug-efficacy later on in the infection is important, since it may also affect treatment efficacy of super-infecting parasite strains, depending on the mechanism. Nevertheless, even though the frequency of drug treatment did not have an impact on parasite densities, drug treatment with a low and high dose on a single day provides support for our hypothesis and shows that reduced drug treatment enhances resistance management while being equally good or better at improving patient health and overall infectiousness.

From an evolutionary point of view, current treatment regimes do not *a priori* make sense as a resistance management strategy. The data presented here confirm this hypothesis. We are not advocating a direct change in malaria patient treatment regimens based solely on these data; more research is needed to this end. However, our data show the importance of evolutionary principles such as competition and selection for the potential spread of resistance and, furthermore, the different impact that various drug treatment regimes exert on resistant parasites. Such principles should not be ignored in policy making. To improve the useful life span of current and future antimalarial drugs, it is key to have an empirically-tested basis for rationally developing patient treatment regimens. Unfortunately, this is currently not the case.

6.6 Appendix

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A mathematical model was utilized in the decision process for choosing drug regimes to test experimentally. The basic model, derived from Mideo et al. (2008), is of the following generalized form:

$$P_S(t+1) = f_S P_S(\ (t), R(t))$$

$$P_R(t+1) = f_R P_R(\ (t), R(t))$$

$$R(t+1) = g(R(t-\tau)) - h_S P_S(\ (t), R(t)) - h_R P_R(\ (t), R(t))$$

where P_S, P_R and R track the daily densities of drug-sensitive parasites, drug-resistant parasites and host red blood cells. The functions f_S and f_D describe the process of red blood cell invasion by parasites as well as the production of progeny parasites within an infected cell. The functions h_S and h_R account for the loss of red blood cells due to infection. In both cases, the subscripts denote the fact that while the functions are the same, they take on unique parameter values for each parasite strain (estimated by maximum likelihood procedures described in Mideo et al. (2008)). The function g describes the daily production of new red blood cells which depends on red blood cell density τ days earlier to account for the maturation time of blood cell precursors. Details of model assumptions, derivations and functional forms are in Mideo et al. (2008).

Drug activity was superimposed on the model described above. Phenomenologically, anti-malarial drug action can be described as operating via a threshold mechanism – above a threshold drug concentration, a given proportion of susceptible parasites are killed and below the threshold, there is no effect of drugs (e.g. White 2008). The length of time that the within-host drug concentration is above this threshold and thus how long the drug-induced parasite decline continues depends on dose, dosing interval and duration of treatment. This was shown to be true for pyrimethamine against *P. chabaudi* in mice (Schneider et al. 2008). Using those data we estimated how drug dose

affects the duration of 'drug activity'. The number of additional days (beyond the inoculation days) of drug activity, a, is given by

$a = 3.557 - \frac{2.586}{1 + \exp[-8.821 + b]}$, where b is the drug dose in mg/kg. We also estimated that each day of drug activity results in a 94% decline in susceptible parasite numbers.

Incorporating this description of drug action into the model of competition required no change to the model structure.

In the absence of drug treatment, the dynamics of the two parasite strains and the host red blood cells are governed by the basic competition model. In the presence of drugs (on days drugs are administered + a days after), the density of drug-sensitive parasites on the next day is a simple linear function of the current density. Thus, $P_S(t+1) = 0.06P_S(t)$, while the rest of the system remains unaltered. Using this approach, we were able to qualitatively capture the outcome of competition experiments between drug-sensitive and drug-resistant malaria parasite clones in the presence of drugs, with conventional drug treatment (a dose of 8mg/kg for 4 days starting at the onset of disease symptoms) and with different initial ratios of sensitive to resistant parasites. This model was used to predict the effects of different drug regimens on the success of the drug-resistant parasite clone within a host. The treatment regimens which were predicted to give the most interesting dynamics and the best potential for suppressing the resistant parasites were included in the experiment.

7. General discussion

A discussion on the specific findings of each experiment is given in the individual chapters. Here, I discuss the general findings of this thesis and the strengths and weaknesses of my experimental approach. Subsequently, I discuss the generalizability and significance of the conclusions drawn in this thesis. I finish by giving recommendations for future work which could improve our understanding of within-host dynamics of malaria parasites and potentially contribute to resistance management.

7.1 Findings

The overall aim of this thesis was to increase our understanding of the within-host dynamics of drug-resistant and drug-sensitive parasites in the presence of drug treatment. A better understanding of the within-host dynamics is important to understand the spread of drug-resistant malaria strains (Hastings 2003; Read and Huijben 2009). The first steps towards this aim were made by studying the competition in untreated and prophylactic drug-treated infections (de Roode et al. 2004). Additional complexity was added through drug treatment at peak parasitaemia when symptoms are occurring (therapeutic treatment) and shortening the treatment course (Wargo et al. 2007). In this thesis, I expanded on these early steps and added more complexity by studying multi-genotype infections, competition at different clone frequencies and competition between resistant and susceptible parasites from the same clonal lineage. Finally, I looked at a variety of different drug treatment regimes.

I observed within-host competitive interactions between drug-resistant and drug-sensitive parasites, with the resistant genotype having a competitive disadvantage in the absence of drug treatment. The frequency of resistant parasites at the start of the infection was an important determinant of the strength of selection: the lower their frequency, the stronger the competitive suppression in non-treated hosts and the greater their competitive release when drugs were given. Resistant and sensitive parasites derived from the same clonal lineage showed similar dynamics following drug treatment as genetically distinct resistant and susceptible parasites. Multiplicity of infection did not have an effect on the within-host dynamics: a larger number of co-

infecting susceptible genotypes did not lead to greater competitive suppression or release of resistant parasites. Lastly, various drug treatment regimes were compared. Conventional drug treatment resulted in the greatest selective advantage for drug-resistant parasites, while less aggressive treatments were equally as effective, or even better, at improving host health and reducing overall infectiousness.

These experiments demonstrate that altering the within-host ecology of drug-resistant parasites by administering drugs and hence removing drug-sensitive competitors results in a positive selection for drug-resistant parasites in various ecological contexts. Together, these data provide proof of principle that drug treatment regimes which are less aggressive than conventional treatment could control drug-resistant parasites to a higher degree than conventional drug treatment, without compromising on other treatment goals.

7.2 Experimental approach

To study the within-host dynamics of resistant parasites and address the questions on optimal treatment regimes, the use of an experimental model system is unavoidable since my objectives cannot be addressed in clinical trials or with human volunteers as we need untreated control groups. Certain choices were made in selecting the experimental approach for this thesis. I will discuss the specificity of my findings towards these choices below.

7.2.1 Plasmodium chabaudi *model*

Although no animal model can fully capture the characteristics and dynamics of *P. falciparum* malaria in humans, the mouse malaria model has proven useful to gain insight in many areas of malaria research, such as malaria immune responses, vaccine development, genetics of drug-resistance and infection dynamics (Wykes and Good 2009). *P. falciparum* and *P. chabaudi* parasites have various features in common. Both species prefer to infect mature red blood cells, as opposed to reticulocytes, and undergo rosetting and sequestration to endothelial cells. *P. falciparum* shows recrudescent asexual peaks after the acute peak, which is also observed in *P. chabaudi* infections. Both species produce similar gametocyte densities in these hosts. Additionally, both infections are normally resolved by the human or mouse hosts

(Taylor-Robinson 1995; Carlton et al. 2001). However, the *P. chabaudi* model does not, of course, capture every biological detail of human malaria.

Mice are not the natural hosts of *P. chabaudi*. In this artificial laboratory model system, *P. chabaudi* is more virulent in mice than *P. falciparum* is in humans. In mice, mortality rates of *P. chabaudi* infections are much higher (5-20%) than *P. falciparum* infections in humans (0.1-1%). Also parasitaemia levels higher than 30% are common among *P. chabaudi* infections, whereas *P. falciparum* infections rarely have a parasitaemia higher than 10%. Since mice are much smaller, total parasite density in mice at peak parasitaemia is around 10^9 parasites (based on a blood volume of 1.5 ml) and in humans 10^{10}-10^{12} (e.g. Hastings and D'Alessandro 2000; Arrow et al. 2004), leading to potentially more mutational variation in *P. falciparum* infections. Mice develop sterilizing immunity against *P. chabaudi* infections, with infections below detection threshold after approximately 50 days, whereas *P. falciparum* infections are usually detected for over 100 days. However, another fundamental difference between *P. falciparum* and *P. chabaudi* parasites is that the former has a life cycle of 48 hours, opposed to 24 hours for *P. chabaudi*. Therefore, in terms of generation time, persistence in the host could be comparable between both species (Taylor-Robinson 1995; Carlton et al. 2001).

Thus, there are several differences between this mouse malaria model and *P. falciparum* in humans. The relative importance of these differences is unknown and depends on the question asked. In this thesis, I am interested in the within-host kinetics of parasites. There are no obvious reasons that these differences should affect my conclusions, with the possible the exception of a higher parasitaemia in *P. chabaudi* infections that could enhance competitive interactions. However, a large body of field data suggests competitive interaction between *P. falciparum* genotypes occurs (Daubersies et al. 1996; Mercereau-Puijalon 1996; Smith et al. 1999; Bruce et al. 2000; Hastings 2003; Talisuna et al. 2006; Bousema et al. 2008; Harrington et al. 2009). Overall, I consider the mouse model system a useful model to study these general principles, though drug treatment policy will not be changed based solely on these data. Nevertheless, these data, along with additional data as described in the future directions section (§ 7.5), could strongly argue for empirical testing in human field trials.

7.2.2 Choice of drug

The antimalarial drug used in these experiments is pyrimethamine. Since I am interested in the effect of drug treatment (that is, the removal of susceptible parasites from a mixed infection) on the relative and absolute fitness of resistant parasites, my conclusions should qualitatively pertain to all drugs. However, some differences between drugs could have a quantitative influence on the questions posed here.

First, the rate at which susceptible parasites are removed can vary for different classes of drugs. Artemisinin-based drugs, for instance, cause a much higher rate of parasite clearance than pyrimethamine (10^4 parasites/cycle and 10^2 parasites/cycle respectively; White 2008), which may have a more immediate effect on the dynamics of drug-resistant parasites. Second, the half-life of antimalarial drugs varies significantly between different drug classes. Pyrimethamine has a relatively long half-life in humans (~ 4 days), compared to artemisinin derivatives which have a very short half-life (~ half an hour; White 1985). Drug half-lives in mice are much shorter: the half-life of pyrimethamine is estimated to be 4.5 hours (Coleman et al. 1986) and that of artemisinin derivatives 19 minutes (Batty et al. 2008). Treatment with a drug that has a long half-life results in an extended period of susceptible parasites being killed compared to drugs that have a short half-life. This will thus result in an extended positive selection on the drug-resistant population. Finally, the genetics of resistance may be of influence on the experimental results since the likelihood of susceptible parasites developing resistance in the course of an experiment may differ per drug. Pyrimethamine resistance develops rather easily in the lab (Walliker et al. 1975), while *de novo* resistance mutations against artemisinins seem to be much rarer (Afonso et al. 2006; Puri and Chandra 2006). Therefore, for some drugs, the possibility of developing resistance in the susceptible parasite population as the experiment progresses has to be taken into account. However, the above differences between drugs should not qualitatively affect the results as described in this thesis. Since the aim here is to study the general principles of removal of drug-susceptible parasites in mixed infections of resistant and susceptible parasites, I do consider it unlikely that the conclusions drawn in this thesis to be specific for pyrimethamine, but clearly other drugs have to be studied.

7.2.3 Density-infectivity analysis

In this thesis, I studied the effects of drug treatment on the relative and absolute fitness of resistant parasites in different ecological contexts. In reality, however, 'true' fitness of the resistant parasites was not studied in these experiments, since the full life cycle of a *Plasmodium* parasite involves a vertebrate and mosquito host. The experiments presented here do not include transmission from mice to mosquitoes because of the additional complexity and number of mice involved. Instead, the fitness of the resistant parasites was inferred by analyzing the gametocyte densities using a density-infectivity function. Such analysis is an improvement of the somewhat arbitrary cumulative gametocyte densities through time, which has been used previously (e.g. Wargo et al. 2007), since mosquito infectivity and hence parasite fitness is not a simple linear function.

This approach, however, is not a perfect alternative for parasite fitness either, because there are many gaps in our knowledge on mosquito infectivity. Apart from the lack of data to determine the exact relationship between gametocyte densities and infectivity as discussed in chapter 2, there are some additional caveats in this type of analysis. The density-infectivity analysis assumes an equal infectiousness through time, whereas in reality, the shape of the density-infectivity function likely changes through time. Much variability in gametocyte infectivity is observed, with for instance gametocytes produced during the acute phase of the infection appearing to be less infectious than gametocytes produced later on (Jeffery and Eyles 1955; Drakeley et al. 2006; Hallett et al. 2006; Wargo 2006 - chapter 3). This initial gametocyte peak, however, is often the highest during the course of infection and weighs heavily towards determining the predicted infectivity of the parasites. Later on in the infection, transmission blocking immunity can also result in reduced infectivity of gametocytes (Beier 1998), while there is also evidence of enhanced gametocyte infectivity of chronic infections (Nedelman 1989; Boudin et al. 1993). Furthermore, the analysis used assumes no interaction between parasites in the mosquito vector, something that is likely to occur in reality (Paul et al. 2002).

Thus, more direct transmission data on gametocyte infectivity is required to refine the density-infectivity analysis, such logistically complex experiments were beyond the scope of this thesis. I do not expect that the conclusions in this thesis are biased due to

the choice of analysis, as the observation is that competitive release of asexual parasites translated into a release of gametocytes which must result in a fitness advantage for resistant parasites, all else being equal. The aim of this analysis was to give a more biological meaningful comparison of predicted infectivity between different treatment groups, not to quantitatively predict actual transmission.

7.3 Generalizability of findings

The general aim of this thesis is to study the effect of drug treatment on the within-host ecology of drug-resistant and drug-sensitive malaria parasites. This aim is based on the main evolutionary principle that the removal of susceptible competitors will lead to a competitive release of drug-resistant parasites (Hastings 2003; de Roode et al. 2004; Wargo et al. 2007; Read and Huijben 2009). This evolutionary principle should be relevant for all diseases where genetically diverse infections are common and resistant pathogens are present and spread through the population.

However, different diseases have different ecological characteristics and may have different treatment goals. Therefore, the optimal treatment regime may vary greatly between diseases. For instance, in the case of cancer, the aim of treatment is not to reduce infectiousness, since it is not an infectious disease. In the case of HIV/AIDS infections, *de novo* resistance mutations, which can threaten the life of the treated patient, are more of a concern than the spread of resistant viruses. Bacterial infections have an additional complexity of lateral transfer of resistance genes for other bacteria. Here, drug treatment increases overall selection for resistance genes, including in the non-target bacterial population, and these resistance genes can subsequently be transferred to the target population.

Thus, while the general evolutionary principles that are demonstrated in this thesis may be applicable to a wide variety of diseases, the application to inform drug treatment regimes may differ considerably between diseases, depending on the social context. Currently, however, the same approach of high-level, long-duration drug treatment is applied to virtually every disease. More empirical data are needed to design disease-specific rational treatment regimes. The current 'kill-them-all approach'

that is used for nearly all diseases is unlikely to be the best resistance management strategy in every specific situation.

7.4 Significance of findings

The translation of these findings to refinement of current treatment protocols for malaria is many steps away. There are some obvious objections to reduce current advised treatment dosages, which I will discuss below.

The first and foremost concern is that anything less than full course chemotherapy could endanger patient health. Yet, the current aim is to kill every single parasite (parasitological cure), which usually involves the continuance of treatment beyond the improvement of patient health (therapeutic cure). The use of therapeutic cure, as opposed to parasitological cure, need not compromise patient health. Further studies need to examine whether reduction in drug dosage from parasitological to therapeutic cure would be enough to control the release of resistant parasites. The implementation of such a treatment regime could be a simple 'take drugs until you feel better' advice, with treatment repeated when symptoms reoccur. Another approach would be a frequent (for instance once a week) low dose of drug treatment to keep parasite growth under control. A further concern to reduced treatment regimes is that lower drug dosages may result in chronic asymptomatic parasitaemia, which can increase the risk of anaemia and other diseases such as chronic hepatosplenomegaly (enlargement of spleen and liver), nephrotic syndrome (kidney damage) and Burkitt's Lymphoma (childhood lymphatic cancer) (Gilles 1986; Greenwood 1987; Wilson et al. 2007). Finally, chronic infections may result in increased gametocyte carriage (Drakeley et al. 2006) and thus increased malaria transmission. On the other hand, chronic malaria infections are known for their generation of protective immunity; removing the majority of the infections from a population could potentially increase malaria morbidity and mortality (Smith et al. 1999; Males et al. 2008).

The above concerns arise from the distinction and possibly the trade-off between resistance management and the other treatment goals: patient health and overall infectiousness. Without a full (empirical) understanding on the effect of various treatment regimes on these three treatment goals (WHO 2006), a conclusion on the

existence of a trade-off between them cannot be drawn. In the best case scenario, the work presented in this thesis, combined with future work leading from this thesis, will lead, through appropriate clinical trials, to the implementation of empirically validated rational treatment design, which will hopefully lead to extending the therapeutically useful life spans of current or future drugs. If future work, either empirically or through clinical trials, demonstrates that current treatment practices are indeed the best practice (based on the three treatment goals), this would still be a valuable outcome: there is a need for an empirically-proven foundation of treatment regimens which are supported by evolutionary principles. If the future teaches us that radical parasitological cure of malaria patients is indeed the best resistance management strategy by strongly reducing the probability of resistance mutations, with selection on pre-existing resistant parasites being an unavoidable side-effect of this treatment strategy, different approaches to slow the spread of resistance, once it has arisen, should be studied. The dramatic effect of increased parasitaemia in pregnant women drug-treated with SP in an area with widespread SP drug resistance was demonstrated in the study by Harrington et al. (2009). Therefore, the best rational treatment regime may depend on the presence of resistant mutants: a reduced treatment regime can be advised to control the spread of these resistant parasites once they have been observed.

7.5 Future directions

Clearly, treatment practices cannot be based solely on the data presented here; more research is needed. First, more experiments are needed to establish the generality of our findings within the *P. chabaudi* model. The conclusions presented in this thesis are based on (i) few clonal parasite lineages in (ii) one inbred host genotype using (iii) one specific drug. While the evolutionary principles are expected to be similar across a wide variety of parasite clones, mice genotypes and immune status, and different classes and combinations of drugs, this hypothesis should be tested empirically before the principles presented here can be tested in clinical trials. Second, experiments using *P. falciparum in vitro* should be conducted to confirm the findings from the *P. chabaudi* experiments. Furthermore, this model can be used to test the various treatment regimes on their ability to reduce the probability of *de novo* mutations, but also selection for resistance. Moreover, resistant mutants from the field could be tested in

this *in vitro* model on their response to different treatment regimes. Third, mathematical models could provide a good insight in the dynamics of resistant and susceptible parasites following treatment and will reduce the number of experimental mice needed. The model used in chapter 6 of this thesis demonstrated the usefulness of such a mathematical model by providing an informed choice of treatment regimes to include in the experiment based on predictions from the model. Such a model, specific for *P. falciparum* would be particularly useful. Additionally, mathematical models could provide insight in the spread of drug-resistance on a population level following various treatment regimes. Fourth, if this thesis, combined with the above proposed future studies, provides enough convincing empirical evidence, the proposed principles could be tested in controlled field trials. During these field trials, much research will need to be done not only on the ecology of resistant parasites but also on the short-term and long-term effects of reduced treatment regimes on patient health and overall transmission.

7.6 Concluding remarks

The conclusions drawn in this thesis are highly controversial because they challenge conventional wisdom. I also recognize that the mouse model, like most other animal models of human disease, fails to capture some aspects of human malaria and I am therefore not advocating an alteration of malaria patient treatment regimens based solely on these data. However, I hope to start a dialogue between policy makers and evolutionary biologists to critically revisit treatment practices and determine what data are needed to lead to better-informed treatment designs. I believe this thesis is a first step on the route towards evolutionary-informed rational patient treatment regimes.

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9. Thesis appendix

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PERSPECTIVE

Evolutionary biology and the avoidance of antimicrobial resistance

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Abstract

Evolutionary biologists have largely left the search for solutions to the drug resistance crisis to biomedical scientists, physicians, veterinarians and public health specialists. We believe this is because the vast majority of professional evolutionary biologists consider the evolutionary science of drug resistance to be conceptually uninteresting. Using malaria as case study, we argue that it is not. We review examples of evolutionary thinking that challenge various fallacies dominating antimalarial therapy, and discuss open problems that need evolutionary insight. These problems are unlikely to be resolved by biomedical scientists ungrounded in evolutionary biology. Involvement by evolutionary biologists in the science of drug resistance requires no intellectual compromises: the problems are as conceptually challenging as they are important.

Introduction

Drug resistance causes immense human suffering globally and is one of the best documented examples of evolution in real time. No self-respecting introductory evolution text fails to mention this, and several professional evolution societies give this as a major practical argument for the teaching of evolution and continued investment in evolutionary science (Meagher and Futuyma 2001). Yet the drug resistance field is – with a few outstanding exceptions – dominated by people with no training in evolutionary biology. Indeed, the microbiologists, clinicians and public health practitioners who publish on drug resistance do not even much use the word 'evolve'– they more naturally use 'emerge', 'spread' or 'arise' (Antonovics et al. 2007). The vast majority of publications on the evolution of antibiotic resistance are in the medical field and not in academic evolutionary biology or genetics journals (Antonovics et al. 2007).

Why do so few professional evolutionary biologists work on drug resistance evolution, particularly given the commercial and grant money involved? This is certainly a specific instance of the remarkable antipathy of most evolutionary biologists to utilitarian science (the journal *Evolutionary Applications* appeared 17 years after the first issue of *Ecological Applications*), an antipathy historians of science have yet to explain. But in the case of drug resistance, the overwhelming volume of data does make assimilating the relevant natural history a challenge, especially as the data are elucidated by physicians, veterinarians, microbiologists and public health specialists, so that a foreign, often pathogen-specific jargon and intellectual culture dominates. Moreover, drug resistance, like many biomedical problems, is perhaps not so interesting to those attracted to evolutionary biology by a passion for 'natural' natural history.

But we think it goes deeper than this. Based on an entirely *ad hoc*, nonrandom sample of our colleagues (largely the evolutionary biologists we meet at conferences), we believe the main reason evolutionary biologists avoid drug resistance is that evolutionary biologists consider drug resistance to be conceptually uninteresting. And at one level it is. As Antonovics et al. (2007) point out, 'the evolution of antibiotic resistance, while critically important from a medical view point, is no longer in and of itself a novel finding in evolutionary biology'. This is true of course, but the evolutionary processes which determine patterns of drug resistance are a different issue. Our straw

poll reveals that most professional evolutionary biologists consider these processes conceptually simple (mutation, selection, fixation), and any solutions largely obvious (combination therapy, reduced drug use). The general feeling seems to be that drug resistance provides excellent examples with which to begin evolution classes and introductory textbooks, and an excellent vehicle to get across basic population genetics. But it is not believed to be an intellectually challenging pursuit around which to structure an interesting evolutionary research program.

Here we attempt to counter this view. We believe there is a strong case for advanced classes in drug resistance evolution, and also that there are numerous conceptually challenging problems in drug resistance evolution to which evolutionary biologists can make unique contributions. The solution of these problems would both be intellectually rewarding and could reduce human suffering. We make this case using malaria, with which we are most familiar, but we believe similar arguments can be made for almost any infectious disease.

We illustrate our case by reviewing a series of fallacies which were, or still are, held by the malaria community (albeit here translated into evolutionary language), and we finish with a number of very open evolutionary research questions. Throughout, we have picked examples which we think are both interesting and challenging, and which demonstrate the practical contribution evolutionary biology is making, or could make, to help alleviate the medical problems caused by drug resistance. By way of an aside, we emphasize that none of this is intended as an argument against fundamental evolutionary research (clearly everything we discuss here builds on that foundation), or a critique of those biologists – evolutionary or otherwise – currently engaging with drug resistance. Our point is that the proportion of evolutionary biologists working on drug resistance is tiny compared with the importance and size of the problem – and the conceptual interest of the issues involved.

The malaria drug resistance problem

Malaria parasites have evolved resistance to all classes of antimalarials that have gone into widespread use, except for the recently deployed artemisinin derivatives (Roll Back Malaria 2008). Resistance was first reported from the field between 1 and 15 years after introduction, depending on the drug (Fig. 1; Peters 1987, Hyde 2005) with drugs failing (i.e. being withdrawn from use by national authorities) years or even decades after that. For instance, chloroquine was widely deployed after the Second World War, with resistance first seen in the field in 1957 in Thailand (Talisuna et al. 2004). Molecular evolution studies show that chloroquine resistance arose only a handful of times, from which it spread world wide (Fig. 2A). It never arose in Africa. Chloroquine was first withdrawn as a first line drug from Thailand in 1973 (Talisuna et al. 2004) and is now recommended only for central America, where parasites are still susceptible (WHO 2008). High level sulphadoxine–pyrimethamine (SP) resistance was observed within the same year as it was introduced in Thailand in 1967 (Talisuna et al. 2004), but replaced chloroquine as first line treatment in most African countries in the early 1990s. Resistance against SP is now widespread. Similar to chloroquine resistance, the major cause of SP resistance in Africa is thought to be a consequence of a selective sweep from a single introduction from southeast Asia (Fig. 2B) (Roper et al. 2004). There may also have been an African origin

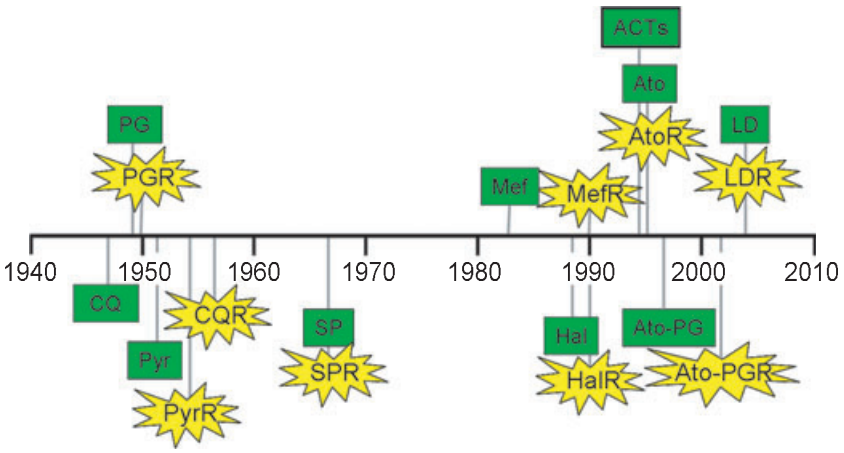

Figure 1 History of the introduction of antimalarial drugs and the first detection of resistance in the field. The following abbreviations are used: CQ, chloroquine; PG, proguanil; Pyr, pyrimethamine; SP, sulphadoxine–pyrimethamine; Mef, mefloquine; Hal, halofantrine; ACTs, artemisinin combination therapies; Ato, atovaquone; Ato-PG, atovaquone–proguanil combination (malarone); LD, LapDap (chlorproguanil–dapsone). R as suffix denotes resistance. Figure redrawn from Hyde (2005).

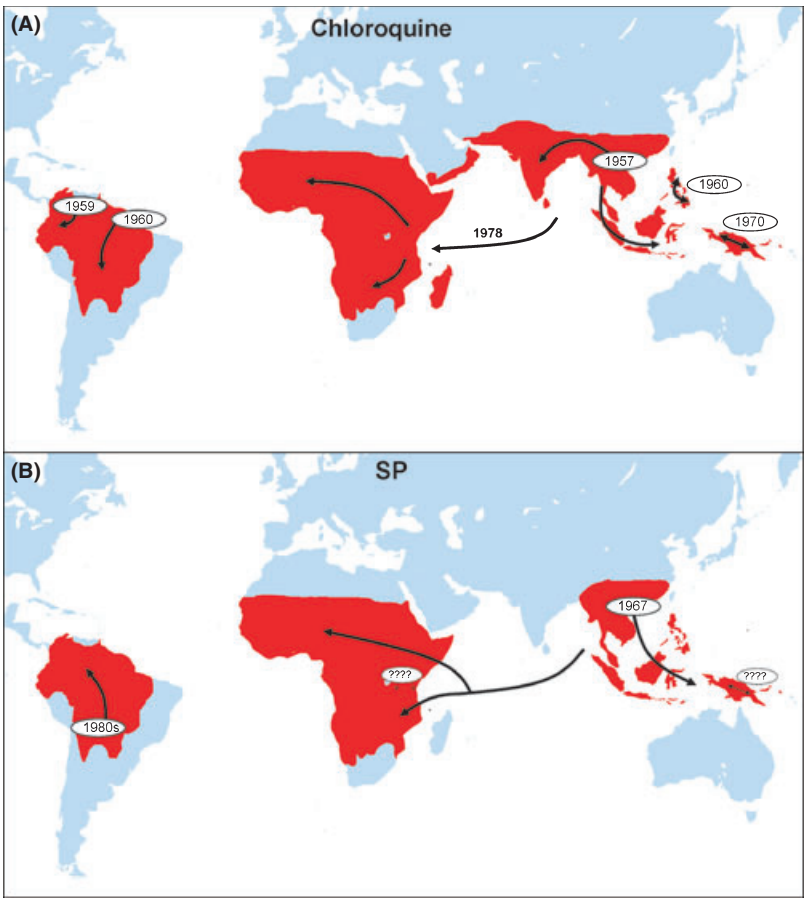

Figure 2 The history of chloroquine and high level pyrimethamine–sulphadoxine (SP) resistance inferred from molecular evolution studies. Chloroquine resistance has spread globally from selective sweeps from five independent origins, none of them in Africa where the health burden of drug resistance is greatest. Resistance to SP is tracked by analyses of the *dhfr* gene which primarily confers resistance to the pyrimethamine component. The timing of two of the independent origins is unclear. SP resistance may have several local origins in Kenya (denoted by '????'), but the majority of SP-resistant infections are a consequence of a selective sweep from a single origin in South East Asia. Figure 2A is redrawn from Wellems (2004), Fig. 2B is a summary of data from Cortese et al. (2002), Nair et al. (2003), Roper et al. (2003, 2004), McCollum et al. (2006, 2007, 2008), Maiga et al. (2007), Mita et al. (2007), Hayton and Su (2008), Saito-Nakano et al. (2008).

in Kenya (McCollum et al. 2006) which seems not to have spread far. Current hopes rest on artemisinin and its derivatives, which have become the key element in current malaria control plans (Roll Back Malaria 2008). Artemisinins are used in co-formulations with other antimalarial agents (artemisinin combination therapy, ACT) in an attempt to minimize the chances of resistance arising. So far this seems to be working, although there are recent reports of parasites with reduced sensitivity to some ACTs (White 2008) and artemisinin resistance can be readily generated in the laboratory (Afonso et al. 2006; Puri and Chandra 2006).

The evolution of drug resistance by malaria parasites is now accepted as inevitable by the World Health Organization (WHO 2006), and a key component of the recently released global malaria action plan (GMAP) is an explicit plan for a drug delivery pipeline – intended as open-ended so long as malaria parasites still exist (Roll Back Malaria 2008). This is the so called 'drug treadmill', the rolling-out of new drugs which will inevitably fail in the face of parasite evolution. The GMAP estimates of the cost of this pipeline are as follows. The basic research budget is estimated as $US34 million per year, and the development cost of bringing a new compound to market at $US250 million over 10 years. The budget for reformulation of compounds already in use (new combinations for instance) is put at $US25 million over 2–6 years. Given the rate at which existing drugs are rendered useless by evolution (in many cases, faster than the speed with which new compounds can go through regulatory processes), and the few useful compounds currently available, GMAP estimates that two new active ingredients for

preventive therapy and six new ingredients for therapeutic use will need to be discovered and brought to market in the coming decade. Over the same period, 14 reformulations of existing and new compounds will need to be developed. Thus, the costs of the pipeline for R&D alone (i.e. excluding the production and deployment costs) will be in excess of $US2.5 billion for the coming decade to get things up to speed. Once the currently inadequate drug arsenal is rebuilt, $US1.5 billion will be required every decade that follows. These are incredible amounts of money for a disease affecting some of the poorest people on the planet.

The challenge for evolutionary biologists is to devise ways to slow the drug treadmill. This includes the demolition of any evolutionarily-naïve medical orthodoxies which drive the treadmill faster. The speed of the treadmill is set primarily by the rate at which mutations conferring resistance escape the clutches of stochastic loss and establish in a population, and then the rate at which they subsequently spread. The WHO considers a drug ineffective once 10% of the parasites in a population have become resistant (WHO 2006, p. 15). Reviews of malaria drug resistance from a population genetics perspective are provided by Hastings and D'Alessandro (2000), Hastings (2001), Koella and Antia (2003), Barnes and White (2005) and Mackinnon (2005), and from the more dominant drug discovery, biochemistry or pharmacokinetic perspective by Hastings et al. (2002), White (2004), Hyde (2005), Barnes et al. (2008), Hayton and Su (2008) and Stepniewska and White (2008). The current WHO guidelines for drug use at national and individual patient levels are published by the WHO (2006).

Fallacy 1: Drugs active against transmission stages slow the spread of resistance

It is apparently conventional wisdom among malariologists that the spread of resistance can be slowed by drugs which target the parasite stages responsible for infecting mosquitoes (sexual stages called gametocytes). For instance Mendez et al. (2002, p. 237) state that 'Antimalarial drugs and drug combinations designed to eliminate both asexual and sexual parasites may deserve priority […] because they will reduce the spread of drug resistance in its earliest stages'. Similarly, the WHO (2006, p. 141) says that 'Reducing transmission is fundamental to the curtailment of drug resistance', and Barnes and White (2005, p. 230) state that '...reducing the carriage of gametocytes […] is necessary to limit the transmission of malaria parasites and the spread of antimalarial resistance'. The intuition behind this orthodoxy is that attacking transmission stages reduces the chances of transmitting a resistant mutant.

But as Hastings (2006a) has pointed out, this argument makes little evolutionary sense. It is true that gametocytocidal drugs will reduce transmission, but they will do so most strongly for sensitive parasites. The relative fitness of resistant and sensitive strains determines the rate of spread of resistance, and this will be increased by drugs targeting transmission stages. Imagine an individual infected with susceptible parasites and a few resistant mutants. If drug treatment kills all susceptible gametocytes, only the few resistant gametocytes will remain. Now imagine treatment with a drug which kills only the replicative (asexual) stages. Susceptible gametocytes remain viable in the blood for weeks, and so will co-occur with the resistant gametocytes. The relative fitness of the resistant mutants is lower in the second scenario compared to the first one. All else being equal, gametocytocidal drugs will enhance the rate at which resistant parasites rise in frequency in a population.

This can turn into a significant effect because small relative fitness differences compound through time. Using a population genetics model, Hastings (2006a) has shown that where drug use is common in a population, the enhanced fitness advantage conferred by gametocytocidal drugs on resistant strains can reduce the useful therapeutic lifespan of a drug by about a year (15%) compared to nongametocytocidal therapy. There may still be sound reasons for using drugs which target transmission stages (e.g. reductions in infectiousness, reducing case incidence, or an incidental side-effect of high lethality against blood stages), but resistance management is not one of them. Indeed, these other reasons need to be weighed against the enhanced resistance evolution that such drugs will prompt.

Fallacy 2: Drugs with long half lives are preferable

Drugs that are slowly eliminated from the body after treatment have several clinical advantages. Clearly, they provide longer term protection against re-infection. For SP, this can be up to 2 months, which in a high transmission region can help prevent novel infections interfering with recovery or generating new symptoms. Slowly eliminated drugs also require fewer administrations to achieve clearance, reducing problems of patient compliance. However, as Watkins and Mosobo (1993) pointed out, drugs with long clearance times also impose stronger selection for drug resistance. This is because, for similar treatment rates, parasites are substantially more likely to encounter drugs with long half lives. If a course of artesunate persists for 5 days, the drug pressure exerted by SP is 10 times greater (Hastings et al. 2002). Drug half life is typically left out of models of drug resistance, yet it may

be one of the most powerful determinants of the useful lifespan of a drug (Hastings et al. 2002). From the resistance management perspective, drugs which are rapidly eliminated from the body are preferable.

An important corollary of this argument is that half lives of drugs used for combination therapy should be similar (Hastings et al. 2002; Hastings and Watkins 2006). The more dissimilar the elimination rates, the greater the chances that resistance to one of the component compounds can become established in a population, thus effectively reducing combination therapy to monotherapy. To achieve clearance with artemisinins alone takes a 7-day treatment regime. Because adherence to a 7-day course is typically poor, the current WHO policy is to combine it with a slowly eliminated antimalarial drug (WHO 2006; White 2008). Recent reports of the failure of these combinations seem to be due to the failure of the partner compound (e.g. Wongsrichanalai and Meshnick 2008). This is likely to continue whenever the partner is a slowly cleared compound. If so, rapid reformulation of ACTs is going to be an open-ended necessity, or there will need to be an abandonment of the aim of complete clearance following ACT.

Fallacy 3: *De novo* resistance mutations are the main enemy

Current malaria treatment guidelines for uncomplicated malaria are radical parasitologic cure (WHO 2006). This is achieved by the administration of sufficiently high and repetitive drug dosages to ensure a kill of every parasite in an infection, and recommended patient treatment regimes are explicitly designed to do this. A major motivator behind this is that '[r]esistance can be prevented, or its onset slowed considerably, by [...] ensuring very high cure rates through full adherence to correct dose regimens' (WHO 2006, p. 12). The underlying reasoning is that complete parasitologic cure (i) reduces parasite biomass and thus the chances of resistance mutations occurring (e.g. White 2004; WHO 2006, p. 165), and (ii) minimizes the number of parasites exposed to sub-curative drug dosages which favor 'tolerant' parasites (e.g. Hastings and Watkins 2006). Tolerant parasites are mutants which are not fully resistant but are able to survive subcurative doses and so are a mutational step towards full resistance (Hastings and Watkins 2006).

However, there are very few data demonstrating that resistance arising *de novo* within a patient is a clinically relevant source of drug failure in malaria patients. Indeed, as we summarized above, the evolutionary history of resistance to two of the major antimalarials, chloroquine and SP, argues that it is effectively zero. Resistance to both drugs arose just a handful of times and spread worldwide (Fig. 2). Indeed, chloroquine resistance seems never to have arisen *de novo* in Africa: it was imported from Asia. So far as is known, every patient in Africa with chloroquine-resistant parasites got them from other people, never from mutational processes within their own infections. Most high-level SP resistance in Africa was similarly due to a single selective sweep of resistance introduced from SE Asia (Fig. 2).

Given this, the widespread conventional wisdom that patients should take a full course of chloroquine to slow resistance evolution makes little sense. Indeed, chloroquine resistance clearly failed to arise in Africa despite widespread underdosing as a consequence of the economically driven noncompliance and low quality drugs (Djimde et al. 1998; Goodman et al. 2007; Bate et al. 2008). Moreover, the recommended patient treatment regimes of overwhelming drug treatment, way beyond what is needed on clinical grounds, imposes the strongest possible selection in favor of resistance, possibly for little clinical gain. Indeed, there is an inconsistency at the heart of the current WHO (2006) guidelines. Correctly, there is a strong argument for reducing unnecessary use of antimalarials at a population level, so as to minimize selection for resistance. In contrast, the recommendation at the single patient level is overwhelming drug use even when there is no clinical need. This maximizes selection for resistance.

De novo resistance may not be irrelevant for all antimalarials, and where single point mutations confer high level resistance as, for example against atovaquone (White 2004), *de novo* mutations may be a serious issue clinically. But for at least high level chloroquine and SP resistance, for which there is the best data on the evolutionary history, resistance arose so rarely that the *de novo* origin of resistance can be ignored as a clinical concern. The explanation for the rare origins is almost certainly because complete resistance with high viability involves multiple mutations (Hastings and Watkins 2006), and so requires a highly unlikely series of mutational events to occur simultaneously. Current combination therapy recommendations are – rightly – designed to make artemisinin resistance similarly unlikely (WHO 2006). When resistance against artemisinins does arise, as it inevitably will, WHO will need to consider patient treatment regimes that will minimize the spread of resistance – not to continue to manage individual malaria patients against the extraordinarily unlikely possibility that every patient will be the source of a second origin. There is no strong argument for treating malaria as if it were a highly mutable pathogen like HIV, and nor is it a bacterium which can easily acquire resistance by lateral gene transfer.

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Fallacy 4: Genetic trade-offs alone determine the magnitude of the costs of resistance

Much circumstantial evidence suggests that resistant malaria parasites have a lower fitness than sensitive parasites in the absence of chemotherapy (Walliker et al. 2005; Felger and Beck 2008). Suggestive evidence for a cost of resistance comes from progressive increases in drug sensitivity in populations where drug use has been discontinued. This has been seen in Malawi (Kublin et al. 2003; Mita et al. 2003; Laufer et al. 2006), Tanzania (Temu et al. 2006), South-Africa (Raman et al. 2008), Thailand (Thaithong et al. 1988), and China (Liu et al. 1995), although there are also areas where a decrease of resistance has not been observed (e.g. McCollum et al. 2007; Yang et al. 2007). Seasonal variation in the frequency of resistant alleles in eastern Sudan and The Gambia is also consistent with costs of resistance. When there is low to no transmission during the dry season, and hence few new malaria cases and essentially no drug use, resistance alleles drop in frequency among the chronically infected patients who source the next outbreak. During the wet season, when high transmission ensures many new disease cases and hence high drug usage, resistance alleles rise in frequency (Abdel-Muhsin et al. 2004; Ord et al. 2007).

As in other pathogens, costs of resistance in malaria presumably arise from the metabolic costs of efflux or detoxification, or reduced biochemical efficiency associated with target site mutations (Hastings and Donnelly 2005); in other words, genetic trade-offs. Most models of malaria drug resistance evolution recognize these costs of resistance, but, if included at all, they are typically taken as a fixed and relatively modest parameter (e.g. a selective disadvantage s, so that the fitness of resistant mutant is $1 - s$, where s in the order of 0.1 or less). Although not much discussed, we believe there is a widely held assumption that these costs can be mitigated by compensatory mutations, as they can be in bacteria and HIV (Levin et al. 2000), so that s can drop further through time. Such selection processes might explain some of the sequential mutational steps associated with chloroquine and SP resistance (Hastings and Donnelly 2005; Hastings and Watkins 2006).

Yet the natural history of malaria makes it highly unlikely that the costs of resistance can be captured by a fixed parameter like 's', and moreover suggests that the costs can often be much larger under some ecologic circumstances. This is because the costs of resistance are a function of the interactions between coinfecting strains within the host. Indeed, this in-host ecology maybe the primary determinant of the magnitude of the costs of resistance. The natural history is as follows.

Human malaria infections frequently consist of more than one *Plasmodium* genotype (Arnot 1998; Babiker et al. 1999; Smith et al. 1999; Bruce et al. 2000; Jafari et al. 2004), so that coexistence of sensitive and resistant parasites is common – and indeed may even be the rule, especially when resistance is beginning to spread through a population. Mixed infections arise from inoculations of genetically diverse parasites by a single mosquito, or contemporaneous bites by multiple mosquitoes infected with different parasites.

A substantial body of correlational epidemiologic evidence is consistent with crowding effects within infections, where population densities of individual genotypes are suppressed when other genotypes are present (Daubersies et al. 1996; Mercereau-Puijalon 1996; Smith et al. 1999; Bruce et al. 2000; Hastings 2003; Talisuna et al. 2006). Direct experimental evidence of crowding cannot be ethically obtained from human infections, but in the rodent malaria model *Plasmodium chabaudi* in laboratory mice, we and others have experimentally demonstrated that strong crowding effects occur. Replicative and transmission stage densities of individual clones within an infection are severely suppressed when coinfecting strains are present (e.g. Jarra and Brown 1985; Taylor et al. 1997; de Roode et al. 2004, 2005; Bell et al. 2006; Wargo et al. 2007). Competitive suppression within hosts also substantially reduces transmission of individual clones to mosquitoes (de Roode et al. 2005). Therefore, the removal of sensitive strains by chemotherapy leads to competitive release of resistant strains (de Roode et al. 2004; Wargo et al. 2007).

We have found in our experiments with rodent malarias that differences in clone performance are greatly magnified by this crowding effect. An example is given in Fig. 3. Pyrimethamine-resistant and sensitive clones are shown. Alone, the resistance clone produces fewer transmission stages. However, when the two clones coinfect the same host, the difference is amplified by clonal competition. We are currently doing experiments to see whether this competitive disadvantage increases as more sensitive coinfecting strains are added. In high transmission regions, infections can consist of five or more clones; if crowding increasing with the number of clones, the fitness disadvantage of resistance could substantially increase with the force of infection.

Thus, the within-host ecology is likely to be a primary determinant of the strength of selection of any resistant mutant in the absence of chemotherapy: the ecologic circumstances can magnify fitness differences way beyond those due simply to ecology-independent genetic trade-offs (Hastings and D'Alessandro 2000; Mackinnon 2005; Hastings 2006b). Except perhaps where single clone infections dominate (as can be the case in low transmission

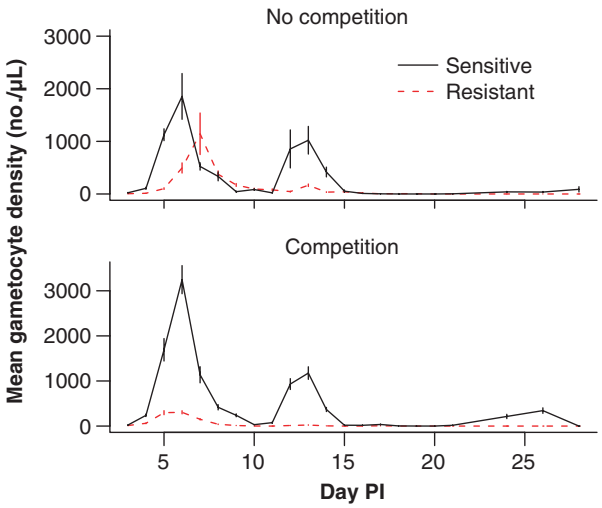

Figure 3 An example of how competition between parasites within infections magnifies differences in performance between sensitive and resistant parasite lines. Top panel – the performance of the two clones when in separate infections. Bottom panel – the performance of the two clones together in the same infection. The lower performance of the resistant clone is further lowered by competition. Plotted points are the mean (±SEM) density of transmission stages of *Plasmodium chabaudi* in peripheral blood through time from three to six laboratory mice per group (S. Huijben, A. R. Wargo, B. H. K. Chan, D. Drew, A. F. Read, unpublished data). Parasite densities were quantified by real time quantitative RT-PCR (Drew and Reece 2007).

regions; Arnot 1998), infection context is almost everything. Within-host genetic diversity is in turn determined by the epidemiology (force of infection), since this is what determines frequency of mixed infections in a population. We suggest that standard population genetics approaches to model drug resistance are likely to be of very limited value in malaria. Explicit evolutionary epidemiologic models (Restif 2009) are needed instead. They are in their infancy in this context (e.g. Hastings 2006b), but there is no escaping this complexity: the epidemiology determines the strength of selection and hence the evolution, and the evolution in turn determines the epidemiologic dynamics.

A highly contentious and unorthodox possibility is suggested by the above considerations (Wargo et al. 2007). Crowding by drug sensitive parasites will suppress transmission stage densities of resistant parasites in untreated hosts. This suggests it might be possible to harness these within-host dynamics for human benefit: the use of patient treatment regimes which do not remove all the sensitive parasites may restrict the transmission of resistance. Some evidence that this might be feasible comes from one of our experiments with rodent malaria (Wargo et al. 2007). We found that treating mice with half the normal dose of antimalarials alleviated the symptoms as effectively as a full dose, but a degree of in-host competition was retained, with the consequence that the transmission potential of the resistant clone was significantly less than in mice given standard doses. Considerably more work needs to be done to evaluate the merits of abandoning the parasitologic cure orthodoxy which currently form the basis of WHO (2006) patient treatment guidelines, but we note that overwhelming chemotherapy is also the way to most effectively up-select resistant mutants in laboratory settings (e.g. Peters 1987), and that host immunity can very effectively clear parasites, especially following drug treatment (Cravo et al. 2001). The theoretical and experimental analysis of the possibility of optimizing patient treatment regimes with respect to *both* patient health and resistance management is long overdue. For instance, would the best regime actually be what is currently considered heretical: take drug treatment until the patient feels better, then further treatment if there are any symptom-associated relapses?

Fallacy 5: Fixation of resistance is inevitable if drug pressure is maintained

A very interesting implication of in-host competition is that the costs of resistance must be frequency-dependent. When a resistant mutant first becomes established in a population, it will typically share its host with competitively more able sensitive strains. As resistance becomes more frequent, resistant strains will increasingly share their hosts with other resistant strains. Because competition magnifies differences in competitive ability as described above, this means that the costs of resistance will be highest early in the spread process, and will decline as resistant strains are increasingly likely to be competing with strains with similar competitive abilities.

Moreover, the benefits of resistance will be similarly frequency dependent. For malaria, the benefits of resistance accrue from two sources: (i) improved survival in a drug-treated host, and (ii) removal of competitors (Hastings and D'Alessandro 2000; de Roode et al. 2004). This competitive release, whereby the resistant clone is able to expand into 'niche space' emptied by chemotherapy has the potential to greatly magnify the survival benefits of resistance – and indeed, it can shorten the therapeutically useful lifespan of a drug many-fold below that expected if resistance evolution were powered only by the survival advantage (Hastings and D'Alessandro 2000). Direct evidence of competitive release cannot be ethically obtained for humans, but in rodent malaria infections it is seen following both prophylatic and therapeutic chemotherapy (Fig. 4; de Roode et al. 2004; Wargo et al. 2007). As this potent selective advantage arises only when a resistant clone is in a coinfection with sensitive clones, it will become progressively weaker as resistance spreads in a population.

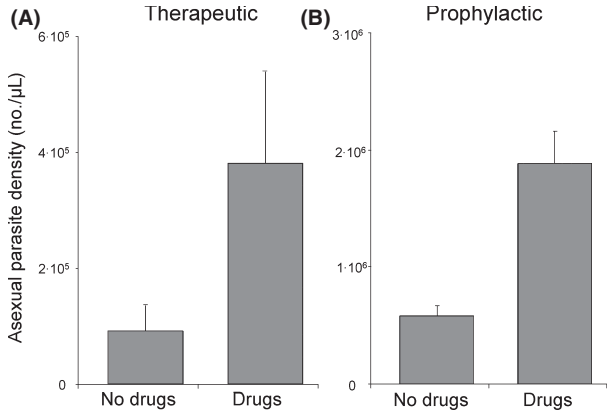

Figure 4 Competitive release of resistant parasites following the removal of sensitive competitors by chemotherapy. The total number of resistant parasites present in infections where sensitive parasites have been removed by drug treatment or allowed to remain (no drugs) are shown. Plotted points are the mean (±SEM) cumulative total number of *Plasmodium chabaudi* parasites present in peripheral blood of mice, based on three to five infections per group. Therapeutic treatment is applied when the hosts first start to show symptoms of malaria (weight loss, anemia); prophylactic treatment is applied at the time host are infected. Data from Wargo et al. (2007), and de Roode et al. (2004) respectively.

Thus, both the costs and benefits of resistance depend on the frequency of resistance in a population. Hastings (2006b) has pointed out that this means that resistance evolution is likely to have dynamics which are considerably more complex than the standard S-shaped curve of rising allele frequencies through time seen in introductory population genetics textbooks. He suggests that this might explain why, for several different countries and drugs, resistance has apparently stabilized at frequencies well short of fixation (e.g. over 8 years in eastern Sudan, chloroquine resistance fluctuated seasonally around an apparently stable equilibrium frequency of 40%; Babiker et al. 2005).

More generally, given the enormous regional and seasonal variation in the force of infection, which is the major determinant of the genetic diversity of malaria infections (Arnot 1998), it may be that there are profound regional differences in the patient treatment regimes and drug deployment strategies which are optimal for resistance management. Existing global recommendations (WHO 2006) may be too simplistic.

Open questions

In addition to the unresolved issues which arise in the context of the preceding fallacies, there are a very large number of other open issues which seem to us unlikely to be resolved without the input of professional evolutionary biologists. Consider, for example, the following:

1 *Why did resistance to chloroquine and SP become established so rarely when resistance spread so globally?* In particular, why is so much drug resistance arising in Southeast Asia? There are at least five hypotheses (Klein et al. 2008), most of which focus on the observation that drug resistance seems to have arisen in areas of low or unstable transmission (White and Pongtavornpinyo 2003).

2 *Will vector control enhance the spread of drug resistance?* Historically, resistance seems to have emerged predominantly in low-transmission areas and spread more effectively in low transmission areas (White 2004; Klein et al. 2008). The large-scale deployment of bednets envisaged by GMAP is aimed at reducing malaria transmission (Roll Back Malaria 2008). Will this lead to more rapid resistance evolution, and necessitate a faster drug pipeline? The influence of transmission rate on resistance evolution has been hotly debated. Several arguments have been put forward that high transmission intensity promotes the spread of drug-resistance. Clonal diversity in infections is higher, exacerbating benefits of resistance, as discussed above. Higher transmission also means that, for a given level of drug use, more parasites will be exposed to drug selection (Mackinnon and Hastings 1998). On the other hand, genetically diverse infections will generate more outcrossed progeny infections, and will thereby lead to the destruction of multi-locus resistance genotypes (Talisuna et al. 2004; Mackinnon 2005). Moreover, areas with low transmission intensity typically harbor fewer immune individuals, who have (i) a higher parasite biomass and so more mutations (White and Pongtavornpinyo 2003), (ii) more symptomatic infections, and hence stronger drug selection (Talisuna et al. 2004; Mackinnon 2005), and (iii) a reduced capacity to clear drug-resistant parasites (Cravo et al. 2001). How these and other conflicting forces play out has yet to be established.

3 *Is the WHO-recommended radical parasite cure really optimal for either patient treatment or resistance management?* We have already questioned above whether radical parasite cure really is the best way of both treating patients and managing resistance evolution. Analyses of the question could also consider the following. In high transmission regions, where people receive more than one infective bite per day (Arnot 1998; Beier et al. 1999; Hay et al. 2000), does radical cure of a symptomatic infection have a sufficiently large clinical beneficial effect to offset the greatly enhanced exposure of parasites to drugs? Does complete parasite clearance make it easier for new parasites to invade?

4 *Will chemotherapy select for more virulent or less virulent parasites?* Chemotherapy could enhance the circulation of more virulent strains by keeping alive patients who would otherwise have died from virulent infections (Gandon et al. 2001; Porco et al. 2005). It could also be that drug tolerance varies with virulence, for instance if more rapidly replicating parasites are more vulnerable to drugs (higher metabolic sensitivity) or less vulnerable (faster population recovery once drug pressure has stopped). For one clonal lineage of a rodent malaria, less virulent parasites were more strongly suppressed by subcurative chemotherapy than more virulent parasites, suggesting that virulence evolution could indeed proceed in parallel with classical resistance evolution (Schneider et al. 2008).

5 *Will the HIV epidemic increase the rate of antimalarial resistance evolution?* There are about 18% more malaria parasites in sub-Saharan Africa as a result of the HIV-associated immunosuppression (Van Geertruyden et al. 2008). Does this increase in parasite number increase the chance of resistance mutations becoming established? If HIV-infection increases the severity of malaria or impairs immune clearance, will drug use become more common, strengthening the selection for resistance?

Coda

We hope that this review of some recent work and ideas in malaria drug resistance has made our general point that, from the perspective of evolutionary science, there is nothing fundamentally uninteresting or easy about drug resistance – and that solutions to the issues could have profound impacts on human health and wellbeing. Evolutionary biologists could conceivably contribute as much as drug discovery specialists (and much more cheaply). It is very hard to imagine that the world will indefinitely fund a malaria drug discovery pipeline at $US1.5 billion per decade, or indeed that there is an unlimited supply of drug classes to be discovered. Using the compounds we already have in the pipeline more effectively is a very high priority. Evolutionary geneticists have and continue to play a crucial role in reconstructing the history of drug resistance (e.g. Fig. 2). The challenge is to add to this an understanding of the processes that shaped this history, and use that understanding to change the future.

For those evolutionary biologists interested in general principles, the issues we have raised here in the context of malaria are relevant across a diverse range of pathogens, from RNA viruses to worms. Our bias is that, at least when it comes to policy and patient treatment, there has been too much focus on simple generalities and not enough focus on the important consequences of disease-specific natural history and indeed location-specific epidemiology. It may also be that considering the drug resistance problem alongside other problems of resistance management, such as mosquito resistance to insecticides, pest resistance to GM crops, and weed resistance to herbicides, would provide novel insights for human health, especially since for some of these, the evolutionary analysis is more advanced (e.g. Labbé et al. 2007), and evolutionary biologists have had a profound impact on policy and implementation on the ground (e.g. Bates et al. 2005).

A major focus of evolutionary biology has been the adaptation of traits where group and individual interests conflict. This way of thinking will undoubtedly prove to be a fertile area in drug resistance too, not least as a guide to the identification of drug targets (Andre and Godelle 2005). But there is also an urgent need to identify resistance management strategies which are good for the group (the currently uninfected, and the patients of the future) without being detrimental to individual patients seeking primary health care right now. In the limit, there is a trade-off between patient treatment and resistance management (the latter being optimized when very few patients are treated with a drug). But such trade-offs are extreme cases. Even where it is necessary to treat effectively large numbers of patients, there are many ways patients can be treated, and among those that similarly restore patient health will be some which are better at resistance management than others. As we have pointed out above, clinical cure is the object of patient treatment, and this need not require parasitologic cure. From a public health perspective, what is the best way to treat patients, impact transmission, and slow the spread of resistance?

More generally, there is a real need to engage with those who deliver and receive health care, and the economists and social scientists who study the process. What sort of resistance management strategies can patients, physicians and public health planners cope with, particularly if they involve an understanding of evolution?

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